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Award Number: DAMD17-02-1-0017

TITLE: Caveolin-1 Modulates Androgen Receptor Signaling in Advanced Prostate Cancer

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REPORT DATE: February 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2005	Final (4 Jan 2002 - 3 Jan 2005)

4. TITLE AND SUBTITLE
Caveolin-1 Modulates Androgen Receptor Signaling in Advanced Prostate Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0017

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

In the past three years, we identified and preliminarily characterized a novel serine/threonine p21 activated protein kinase 6 (PAK6) as the key signal mediator in regulating AR signal transduction within caveolae/raft domain. We also demonstrated a cross-talk between the caveolin-1/AR and PI3 kinase/Akt signal pathway in hormone dependent cell survival. Overall, our results established a biochemical basis on the notion that caveolin expression is associated with prostate cancer progression. The "neoexpression" caveolin in prostate cancer progression represents a gain of function event in cancer survival. These results illustrate the important role of AR non-genomic effect in response to androgen stimulation. Moreover, the identification of a novel serine/threonine PAK6 kinase underscores the relevance of this signal pathway in mediating AR nongenomic effects. Our results support the hypothesis from our original proposal that AR signals from rafts to regulate its transcriptional function. These findings pave the way to further define the underlying signal cross-talk in AR-mediated signaling.

14. SUBJECT TERMS
Androgen receptor, caveolin-1, gene regulation, signal transduction, androgen, transcriptional regulation

15. NUMBER OF PAGES
38

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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DOD Progress Report:

Caveolin-1 Modulates Androgen Receptor Signaling in Advanced Prostate Cancer

Introduction:

The underlying mechanism of the progression of prostate cancer to hormone-independent disease is poorly understood. Neoexpression of caveolin-1, a scaffold protein associated with caveolae membrane microdomains, has been shown to correlate with hormone resistance and metastasis in both human and mouse prostate cancer models (Nasu et al., 1998; Yang et al., 1999). We find that overexpressing caveolin-1 in human prostate cancer cells positively regulates androgen receptor transactivation activity. We identify that modulating caveolin expression levels dramatically alters the sensitivity of AR to androgen stimulation in cellular models (Lu et al., 2002). We hypothesize that caveolin-1 scaffolding signal complex plays a regulatory role in AR activation pathway. Our specific aims are: (1) Mapping the submolecular domains required for AR and caveolin interaction, (2) Functional and biochemical characterization of the AR/caveolin interaction, (3) Characterization of the physiological role of caveolin-1 overexpression in AR signaling of prostate carcinoma cell, and (4) Evaluating the effect of caveolin scaffolding domain CSD peptide in prostate cancer PC3 tumor growth in vivo.

Report Body:

(1) In year one, we focus our effort on proposed work for Specific Aim 2 and 3 of characterizing both the functional and physiological roles of caveolin in AR signaling. Using mammalian two-hybrid assay, we determined two caveolin interacting domain in androgen receptor (AR) that localized to AR N-terminus and ligand binding domain (LBD). The AR binding domain of caveolin was determined to localize to the region (Cav30-60) precedes the caveolin scaffolding domain (CSD) of caveolin. These interactions were verified biochemically by GST-pull down and co-immunoprecipitation. We generated expression vectors which covering various caveolin domain including CSD (caveolin scaffolding domain), Cav(1-30), Cav(1-60), Cav(31-60) and Cav(137-178) using pcDNA3.0 as an expression vehicle . Their effects on AR signaling and cell growth regulation were tested by co-transfecting LN-Cav cells with these expression vectors and reporter genes. Among those peptides tested, we determined that Cav(31-60) peptide to be most effective in perturbing the caveolin/AR interaction. Furthermore, Cav(137-178) was demonstrated to be most effective in interfering with androgen activated PI3kinase/Aktsignal pathway. Our data indicate that disrupting caveolin/AR interaction down-regulates androgen stimulation-induced Akt activation. To further demonstrate the importance of caveolin/AR interaction in promoting cell survival, we have initially characterized a pair of caveolin RNAi oligonucleotide for downregulation of caveolin expression in LAPC4 and PC3 cells. Our data indicate that caveolin knock-down induces apoptosis of these cells. The molecular mechanism of these

(2) In year two, we focus our effort on proposed work for Tasks 2 and 4 characterizing both the functional and physiological roles of caveolin in AR signaling. Using a mammalian two-hybrid assay, we determined two caveolin interacting domains in androgen receptor (AR) that localized to AR N-terminus and ligand binding domain (LBD). The AR binding domain of caveolin was determined to localize to the region (Cav30-60) preceding the caveolin scaffolding domain (CSD) of caveolin (Lu et al., 2001). These interactions were verified biochemically by GST pull-down and co-immunoprecipitation.

Various caveolin domains, including CSD (caveolin scaffolding domain), Cav(1-30), Cav(1-60), Cav(31-60) and Cav(135-178), were cloned into mammalian pcDNA3.0 expression vector. Their effects on interfering with AR/caveolin interaction were tested by a co-transfection study using a previously defined pACT-AR/pBIND-CAV9FL) mammalian two-hybrid assay. As shown in Figure 1, among peptides tested, peptides Cav(31-60) and Cav(135-178) appeared to be most effective in perturbing the caveolin/AR interaction. This is consistent with our previous results that identified Cav(31-60) as an AR interacting domain.

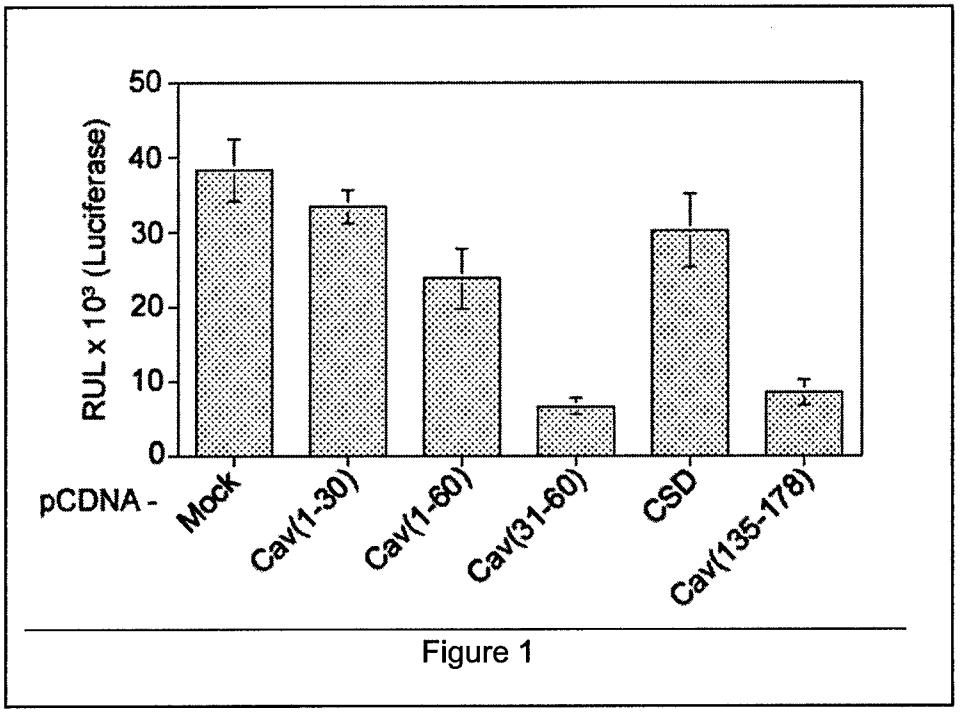

Figure 1

To test the effects of caveolin peptides in modulating androgen-mediated signals, LNCap cells were transiently transfected with various expression vectors encoding the caveolin peptides. 24 hours post-transfection, the level of androgen-stimulated Akt activation was determined by western blot analysis using a Ser-473 phospho-specific antibody (Li et al., 2003). As shown in Figure 2, an increase of Akt Ser-473 phosphorylation was observed in LNCap cells in response to androgen stimulation. While transient transfection with vectors harboring Cav(1-30), Cav(30-60) or CSD does not alter the androgen-induced Akt activation, expression of the Cav(135-178) peptide dramatically downregulates the androgen-

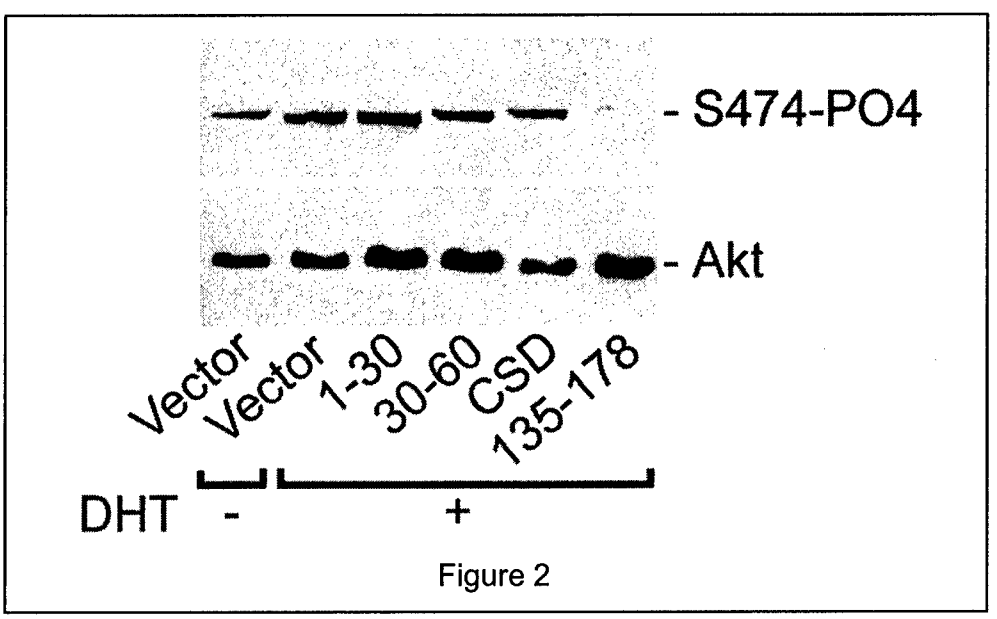

Figure 2

stimulated Akt activation since Cav(135-178) was mapped to be an AR interacting domain. Our data suggest the possibility that down-regulation of androgen stimulation-induced Akt activation may be caused by disrupting caveolin/AR interaction.

To further demonstrate the role of caveolin in promoting prostate cancer cell survival, we have initially characterized a pair of caveolin RNAi oligonucleotides for their ability to downregulate caveolin expression in PC3 cells (Paddison et al., 2002) (gene bank accession number AF070648):
#139-GGUCAGCAGCCUCCCUGAA
#256-CAUCUUUAUCCGUAGUGGG.

As shown in Figure 3, PC3 cells were transiently transfected with 10 nM of scrambled control (SC), #139-, or #256- siRNAs using lipofectamine 2000. 72 hours post-transfection, levels of caveolin expression are determined by a western blot analysis . As shown in Figure 3, a dramatic decrease of caveolin protein level is observed in caveolin specific siRNA #139 and #256. Preliminary results indicate that caveolin knock-down induces apoptosis in these cells (data not shown).

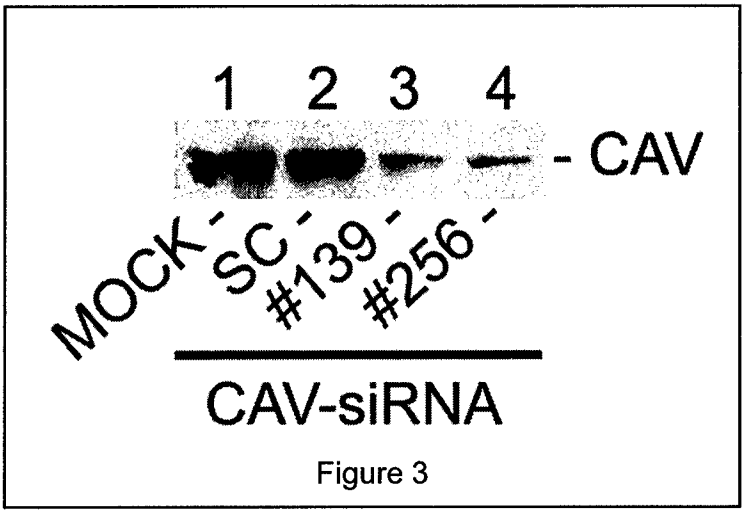

Figure 3

(3) In the final year, we continue focusing our effort on the proposed work for Tasks 3 and 4 characterizing both the functional and physiological roles of caveolin in AR signaling. As partial fulfillment for the goal in Aim 3, in an effort to identify components associated with the AR/caveolin signal complex, we employed a proteomic approach to characterize the raft domain associated anti-AR immune complex. We identified a novel serine/threonine p21 activated protein kinase 6 (PAK6) as one of the signal components involved in the complex. PAK6 was previously identified to be an AR interacting partner by a yeast two-hybrid assay (Lee et al., 2002). To further determine the significance of PAK6 in the androgen receptor-mediated signal transduction, we have preliminarily characterized the molecular mechanism of PAK6 activation. We found that PAK6 can be activated directly via MKK6/P38 MAP kinase pathways. The study results were recently accepted for publication (see Appendix 1). Deregulated P38 kinase was previously identified to be associated with prostate cancer metastatic progression in a TRAMP prostate cancer model (Uzgare et al., 2003). Our identification of PAK6 as a potential regulator of AR signaling provides new insights on future understanding of the androgen/AR regulated non-genomic signals.

The p21-activated kinases (PAKs) contain an N-terminal Cdc42/Rac interactive binding (CRIB) domain, in the group 1 PAKs (PAK1, 2, and 3), which regulates the activity of an adjacent conserved autoinhibitory domain. In contrast, the group 2 PAKs (PAK4, 5, and 6) lack this autoinhibitory domain and are not activated by Cdc42/Rac binding, and the mechanisms that regulate their kinase activity have been unclear. Our study found that basal PAK6 kinase activity was repressed by a p38 MAP kinase antagonist and could be strongly

stimulated by a constitutively active MAP kinase kinase 6 (MKK6), an upstream activator of p38 MAP kinases. Mutation of a consensus p38 MAP kinase target site at serine 165 decreased PAK6 kinase activity. Moreover, PAK6 was directly activated by MKK6, and mutation of tyrosine 566 in a consensus MKK6 site (threonine-proline-tyrosine, TPY) in the activation loop of the PAK6 kinase domain prevented activation by MKK6. PAK6 activation by MKK6 was also blocked by mutation of an autophosphorylated serine (serine 560) in the PAK6 activation loop, indicating that phosphorylation of this site is necessary for MKK6-mediated activation. PAK4 and PAK5 were similarly activated by MKK6, consistent with a conserved TPY motif in their activation domains. The activation of PAK6 by both p38 MAP kinase and MKK6 suggests that PAK6 (and likely other PAKs) have a unique and specialized role in the cellular response to stress.

We have identified two caveolin derived peptides, Cav30-60 and Cav135-178, that downregulate AR-mediated signaling in a co-transfection assay To test these peptides directly in cell culture (or latter in the animal model), we are in the process of generating cell permeable caveolin peptide by fusing an HIV-tat sequence to the inhibitory caveolin peptides. In this aspect, we have generated expression vectors that fuse an HIV-Tat cell permeable sequence (Becker-Hapak et al, 2001) with caveolin-derived peptides Tat-Cav30-60 and Cav 135-178. We are currently in the process of characterizing the expression in bacteria and small-scale purification of the fusion peptides.

Key Research Accomplishments:

1. Identified AR sub-molecular domains, AR-N and LBD, interacting with caveolin
2. Identified caveolin sub-molecular domain, CSD, interacting with AR
3. Determined the biological effects of various caveolin fragments on AR mediated signaling.
4. Established the physiological interactions between AR and caveolin-1 at both molecular and biochemical levels.
5. Characterized the feasibility of using small interference RNA (siRNA) in caveolin expression knock-down study in tissue culture model.
6. Identified the new signal component PAK6 as a key mediator of AR/caveolin interaction related signals.
7. Characterized the feasibility of using HIV-Tat fusion caveolin derived peptides in perturbation of AR-mediated signals.

Reportable Outcomes:

1. Publications:

R. Kaur R, Liu X, Gjoerup O, Zhang A, Yuan X, Balk SP, Schneider MC and Lu ML. Activation of p21 Activated Kinase 6 (PAK6) by MAP Kinase Kinase 6 and p38 MAP Kinase. J. Biol. Chem., 2005, in press. (Appendix 1)

Freeman MR, Cinar B and Lu ML. Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer. Trends Endocrinol. Metabol. Rev., 2005, in press. (Appendix 2)

2 Cell Lines:
LNCap cell stably expressing caveolin, LNCav

3. Expression Vectors:
Mammalian expression vectors for AR, caveolin expression. pcDNA-AR. pcDNA-Cav, pVp-AR, pcDNA-AR. pcDNA-Cav(1-30), Cav(31-60), Cav(137-178).
Bacteria expression vectors encoding fusion protein of Tat-Cav 30-60 and Tat-Cav135-178.
4. Human caveolin 1 specific Small interference RNAs.

Conclusion:

In summary, we have been very productive in the past three years on this project. Two manuscripts that describe our findings have been accepted for publication in peer-reviewed journals. Three additional manuscripts are in preparation for publication to report our yet newer findings on the project. We anticipate finishing these studies in the coming year. Although we have accomplished most of the goals depicted in our original proposal in a timely fashion, some unexpected technical hurdles delayed our completion of the entire project.

In the past three years, we identified and preliminarily characterized a novel serine/threonine p21 activated protein kinase 6 (PAK6) as the key signal mediator in regulating AR signal transduction within caveolae/raft domain. We also demonstrated a cross-talk between the caveolin-1/AR and PI3 kinase/Akt signal pathway in hormone dependent cell survival. Overall, our results established a biochemical basis on the notion that caveolin expression is associated with prostate cancer progression. The "neoexpression" caveolin in prostate cancer progression represents a gain of function event in cancer survival. These results illustrate the important role of AR non-genomic effect in response to androgen stimulation. Moreover, the identification of a novel serine/threonine PAK6 kinase underscores the relevance of this signal pathway in mediating AR nongenomic effects. These findings pave the way to further define the underlying signal cross-talk in AR-mediated signaling.

Our results support the hypothesis from our original proposal that AR signals from rafts to regulate its transcriptional function. Because of the known tendency of the raft compartment(s) to sequester signal transduction complexes, we believe this subcellular location represents a rich potential source of new information on the role of the AR in nongenomic signaling. Although the significance of nongenomic steroid hormone actions in cancer is still controversial and is being actively debated, the well-known loss of hormonal

control of the AR during PCa progression provides a fertile and clinically relevant topic area for investigating these potentially important signaling mechanisms.

References:

1. Lu M.L., Schneider M.C., Zheng Y., Zhang X. and Richie J.P. J. Biol. Chem. **276**, 13442 (2001).

2. Li L., Cheng H.R., Tahir S.A., Ren C. and Thompson T.C. Mol. Cell Biol. **23**, 9389. (2003).

3. Paddison P.J., Caudy A.A., Bernstein E., Hannon G. J. and Conklin D.S. Gene & Dev. **16,** 948 (2002)

4. Uzgare A.R., Kaplan P.J. and Greenberg NM. Prostate. **55**,128 (2003).

5. Lee SR, Ramos SM, Ko A, Masiello D, Swanson KD, Lu ML, Balk SP. Mol. Endocrinol. **16**:85 (2002)

6. Becker-Hapak M, McAllister SS, Dowdy SF. Methods, **24**:247-56 (2001)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 5, Issue of February 4, pp. 3323–3330, 2005
Printed in U.S.A.

Activation of p21-activated Kinase 6 by MAP Kinase Kinase 6 and p38 MAP Kinase*

Received for publication, June 16, 2004, and in revised form, November 9, 2004
Published, JBC Papers in Press, November 18, 2004, DOI 10.1074/jbc.M406701200

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The p21-activated kinases (PAKs) contain an N-terminal Cdc42/Rac interactive binding domain, which in the group 1 PAKs (PAK1, 2, and 3) regulates the activity of an adjacent conserved autoinhibitory domain. In contrast, the group 2 PAKs (PAK4, 5, and 6) lack this autoinhibitory domain and are not activated by Cdc42/Rac binding, and the mechanisms that regulate their kinase activity have been unclear. This study found that basal PAK6 kinase activity was repressed by a p38 mitogen-activated protein (MAP) kinase antagonist and could be strongly stimulated by constitutively active MAP kinase kinase 6 (MKK6), an upstream activator of p38 MAP kinases. Mutation of a consensus p38 MAP kinase target site at serine 165 decreased PAK6 kinase activity. Moreover, PAK6 was directly activated by MKK6, and mutation of tyrosine 566 in a consensus MKK6 site (threonine-proline-tyrosine, TPY) in the activation loop of the PAK6 kinase domain prevented activation by MKK6. PAK6 activation by MKK6 was also blocked by mutation of an autophosphorylated serine (serine 560) in the PAK6 activation loop, indicating that phosphorylation of this site is necessary for MKK6-mediated activation. PAK4 and PAK5 were similarly activated by MKK6, consistent with a conserved TPY motif in their activation domains. The activation of PAK6 by both p38 MAP kinase and MKK6 suggests that PAK6 plays a role in the cellular response to stress-related signals.

p21-activated kinases (PAKs)[1] were originally identified as serine/threonine protein kinases that bound to and were activated by the p21 GTPases, GTP-Cdc42 and -Rac. Binding of p21 is mediated by an N-terminal Cdc42/Rac interactive binding (CRIB) domain, and biochemical and crystal structure analyses of PAK1 have shown that the CRIB domain regulates the inhibitory activity of an adjacent autoinhibitory domain (AID). In the absence of p21 GTPase binding, PAK1 exists as an autoinhibited dimer in which the N-terminal AID of one PAK1 molecule in the dimer binds to the other catalytic domain and blocks its function. Binding of GTP-Cdc42 or -Rac causes the AID to dissociate from the catalytic domain and activates its kinase activity, with subsequent phosphorylation of sites in the N-terminal regulatory domain and in the activation loop of the kinase domain serving to maintain the activated state (1, 2).

The N-terminal CRIB domain and AID are highly conserved in human PAK2 and PAK3, and these PAKs have been categorized with PAK1 as group 1 PAKs. PAK6 was initially identified in yeast two-hybrid screens for androgen receptor-interacting proteins (3, 4). PAK6 has a C-terminal kinase domain with homology to the group 1 PAKs and an N-terminal CRIB domain. However, PAK6 lacks the conserved AID and is not stimulated by ligation of its CRIB domain, which binds selectively to GTP-Cdc42 (3). Human PAK4 and PAK5 similarly lack the conserved AID and along with PAK6 comprise the group 2 PAKs (5).

Group 1 PAKs (PAK1, PAK2, and PAK3) are involved in the regulation of diverse cellular processes such as cell motility, morphology, cytoskeletal reorganization, and gene regulation. Much less is known about the regulation and function of group 2 PAKs (PAK4, PAK5, and PAK6). PAK4 is expressed ubiquitously, and activated PAK4 has been shown to mediate cytoskeleton reorganization and filopodia formation (6, 7). Targeted disruption of PAK4 results in embryonic lethality. PAK5 is highly expressed in brain and neuronal tissues and has been shown to promote neuron outgrowth during development. RNA blot analyses have shown that PAK6 is expressed most highly in brain and testes and at lower levels in multiple tissues including prostate and breast. In transfection studies, PAK6 has been shown to suppress androgen receptor transcriptional activity and similarly bind to and repress estrogen receptor (3).

The mechanisms that regulate the group 2 PAKs are unclear, but the absence of a conserved AID indicates that the modes of regulation differ from the group I PAKs. In the current study, we describe a novel mechanism of PAK6 regulation by the MKK6-p38 MAP kinase pathway. Our results demonstrate that MKK6 activates PAK6 by targeting two separate sites, a consensus p38 MAP kinase substrate site (Ser-165) and a tyrosine (Tyr-566) in the activation loop of the kinase domain. Significantly, this tyrosine is part of an MKK6 substrate motif (threonine-*X*-tyrosine) that is conserved in the group 1 and 2 PAKs but is otherwise largely restricted to activation loops of MAP kinases, where it undergoes direct dual phosphorylation by MAP kinase kinases. This study further shows that MKK6-mediated activation does not alter the autophosphorylation of a regulatory serine in the activation loop of PAK6 (Ser-560), which is also conserved in the activation loop of all PAKs.

* This work was supported by National Cancer Institute Grant CA8799701 and Department of Defense Grant DAMD17-02-1-0017. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PAK, p21-activated kinase; MKK6, MAP kinase kinase 6; CRIB, Cdc42/Rac interactive binding domain; AID, autoinhibitory domain; MKP-1, mitogen kinase phosphotase-1; WT, wild type; HA, hemagglutinin; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IP, immunoprecipitation; mAb, monoclonal antibody.

Moreover, this serine is required for MKK6-p38 MAP kinase activation of PAK6. Taken together, the results in this study indicate that PAK6 is regulated by MKK6 and p38 MAP kinase and that the PAK6 activation loop is regulated by both MKK6 and autophosphorylation.

EXPERIMENTAL PROCEDURES

Materials and Reagent—p38 MAP kinase inhibitor SB203508 and MEK1 inhibitor PD98059 were purchased from LC Laboratories (Woburn, MA). cAMP-dependent protein kinase activator forskolin and histone H4 were purchased from Sigma. c-Jun NH_2-terminal kinase inhibitor SP600125, PI3K inhibitor LY 294002, and MEK1 inhibitor U0126 were purchased from Calbiochem. Protein-A-conjugated Sepharose beads were from Amersham Biosciences. Monoclonal antibodies against phosphotyrosine and p38 MAP kinase were from Upstate Biotechnology (Lake Placid, NY), and monoclonal antibody 12CA5 against the hemagglutinin (HA) tag was from Berkeley Antibody (Berkeley, CA). Antiserum against phospho-PAK4(Ser-474)/PAK5(Ser-602)/PAK6(Ser-560) was purchased from Cell Signaling Technology (Burlington, MA). The QuikChange mutagenesis kit was from Stratagene Inc. (La Jolla, CA). Anti-PAK6 polyclonal antiserum was generated against glutathione *S*-transferase fused with PAK6 residues 115–383.

Expression Vectors and Constructs—PAK6 was cloned in-frame with an N-terminal HA-tag containing pcDNA3.0 vector (Invitrogen). N-terminal tagged MKK6(EE) was a gift from Dr. R. Davis (8). Generation of S165A and Y566F point mutations were done by PCR-based site-directed mutagenesis QuikChange kit following the manufacturer's instructions. The mutation primers used for S165A are: CCG TGG CCC GAG CCA CAG GAA CCA CGG GTC CTG CCC AAT GGG and CCG TGG CCC GAG CCA CAG GAA CCA CGG GTC CTG CCC AAT GGG. The primers used for Y566F are: TCC CTG GTG GGA ACC CCC TTC TGG ATG GCT CCT GAA GTG and CAC TTC AGG AGC CAT CCA GAA GGG GGT TCC CAC CAG GGA. The primers used for S560A (S560D or S560E) are: GAC GTC CCT AAG AGG AAG GCC (or GAC (for D) or GAA (for E)) CTG GTG GGA ACC CCC TAC and GTA GGG GGT TCC CAC CAG TTC (or GTC (for D) or TTC (for E)) CTT CCT CTT AGG GAC GTC. The site-directed mutagenesis was performed based on the manufacturer's protocol.

Cell Culture and Transient Transfection—HEK293 cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with antibiotics and 10% fetal bovine serum. The cells were transfected by electroporation with a total of 10 μg of plasmid DNA using a Gene Pulser from Bio-Rad.

Gel Electrophoresis and Immunoblotting—The proteins were separated by SDS-PAGE with a standard reducing protocol. Following electrophoresis, the proteins were electroblotted to a nitrocellulose membrane. The protein bands were visualized by Ponceau S red staining. The blots were blocked by 5% nonfat dry milk, 0.05% Tween 20, and 1% bovine serum albumin in Tris-buffered saline (10 mM Tris, pH 8.0, 135 mM NaCl). Immunoblotting was performed with designated antibodies and visualized with an ECL detection system (Pierce) following the manufacturer's protocol.

Immunoprecipitation—Immunoprecipitation of PAK6 and proteins containing phosphotyrosine was employed following a standard protocol. In brief, the cells were lysed in immunoprecipitation radioimmune precipitation assay buffer containing 50 mM Tris, pH 7.4, 135 mM NaCl, 1% (v/v) Triton X-100, 0.25% (w/v) deoxycholate, and 0.05% (w/v) SDS and supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 mM diisopropyl fluorophosphate, 5 μg/ml pepstatin, 1 mM EDTA). The lysates were cleared by centrifugation at 12,000 × *g* for 30 min at 4 °C. The supernatants were incubated with individual antibodies (1 μg) and protein A-Sepharose beads (20 μl of packed beads) at 4 °C for 1 h. At the end of incubation, the beads were washed five times with lysis buffer. The resulting immunoprecipitated immunocomplexes were solubilized in 40 μl of Laemmli sample buffer, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. The protein complex was detected by Western blot analysis and developed by ECL (Pierce; Supersignal).

In Vitro Kinase Assay—Kinase reactions of immunoprecipitated PAK6 were performed in kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 2 mM dithiothreitol, 200 μM ATP) supplemented with 2.5 μg/reaction of histone H4 and 20 μCi/reaction of radioactive ATP. The reactions were incubated for 30 min at 30 °C and stopped by the addition of sample buffer containing SDS. The reactions were resolved by SDS-PAGE, and autoradiography of radiolabeled protein was performed.

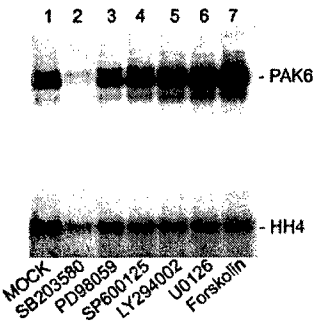

FIG. 1. **Inhibition of PAK6 kinase activity by p38 MAP kinase inhibitor SB203580.** Various agents were used to treat HEK293 cells transiently transfected with HA-PAK6 for 24 h. The cells were treated with pharmacological agents for 1 h before being subjected to the immunoprecipitation protocol. PAK6 was immunoprecipitated from cell lysates with anti-HA mAb 12CA5, and its kinase activity was assayed in the presence of histone H4 and [γ-^{32}P]ATP. Both autophosphorylation and substrate phosphorylation were analyzed after SDS-PAGE and autoradiography. The *upper panel* shows the autophosphorylation of PAK6. The *lower panel* shows the phosphorylation of exogenously added histone H4 substrate. The dosages used were: SB203580, 25 μM; PD98059, 100 μM; SP600125, 10 μM; LY294002, 20 μM; U0126, 50 μM; and forskolin, 10 μM.

RESULTS

PAK6 Is Inhibited by p38 MAP Kinase Antagonist—In contrast to PAK1, PAK6 exhibits readily detectable basal kinase activity even in the absence of exogenous stimulation. To determine the molecular mechanisms that regulate PAK6 kinase activity, we tested a group of agents with known specificity either as inhibitors or as activators of their respective pathways. HEK293 cells were transiently transfected with HA-tagged PAK6 for 24 h and then treated with drugs for 1 h prior to immunoprecipitation with an anti-HA antibody. Kinase activities in the immunoprecipitates were then measured by *in vitro* kinase assays, using histone H4 as an exogenous substrate. Among nine tested agents, only the p38 MAP kinase inhibitor SB203580 exhibited inhibitory effects on PAK6 kinase activity, with reduced autophosphorylation and reduced phosphorylation of the exogenous histone H4 substrate (Fig. 1).

PAK6 Is Activated by p38 MAP Kinase Upstream Activator MKK6—Inhibition of PAK6 kinase activity by SB203580 suggested that the p38 MAP kinase pathway was regulating PAK6 activity. To further test this possibility, we co-transfected HEK293 cells with HA-tagged PAK6 and a constitutively active MKK6, MKK6(EE), an upstream activator of p38 MAP kinase (9, 10). The effect on PAK6 kinase activity was then assessed by *in vitro* immunoprecipitation kinase assays. As shown in Fig. 2*A*, MKK6(EE) caused an increase in PAK6 autophosphorylation and histone H4 phosphorylation. Immunoblotting of the immunoprecipitates with the anti-HA antibody confirmed that PAK6 protein expression was not altered, indicating that MKK6(EE) increased PAK6 kinase activity (Fig. 2*B*).

The involvement of p38 MAP kinase in this PAK6 activation by MKK6(EE) was examined by assessing the inhibitory effect of SB203580. Significantly, although SB203580 markedly down-regulated PAK6 activity in the absence of MKK6(EE), it only partially inhibited the MKK6(EE)-induced activation of PAK6 (Fig. 2*C*). This partial inhibition was consistent with the high level of p38 MAP kinase activation in the MKK6(EE)-transfected cells (Fig. 2*D*). However, the substantial PAK6 activation in the MKK6(EE)-transfected and SB203580-treated cells also suggested a p38 MAP kinase-independent mechanism for PAK6 activation.

To further address the role of p38 MAP kinase in PAK6 activation, we attempted to identify a site that was phospho-

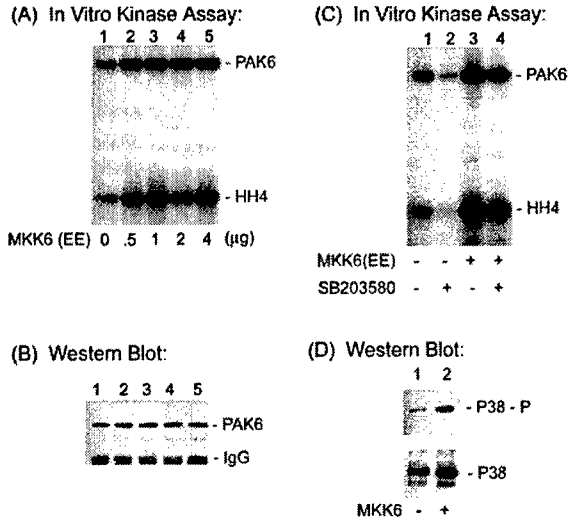

FIG. 2. **MKK6-induced PAK6 activation is down-regulated by SB203580.** *A*, 293 cells were transiently co-transfected with both HA-PAK6 and various dosages of constitutively active mutant MKK6(EE). *In vitro* kinase activity was assessed by a kinase assay on the immunoprecipitated PAK6. *B*, equal amounts of PAK6 from the immunoprecipitation in *A* were immunoblotted with anti-HA. *C*, the effect of SB203580 on MKK6-activated PAK6 kinase activity was measured by an IP/kinase assay using cell lysates of 293 cells transiently co-transfected with MKK6 and PAK6 that were treated with or without SB203580. *D*, the phosphorylation status of p38 MAP kinase in response to MKK6 activation was confirmed by Western blot analysis using an anti-p38 phospho-specific antibody (*upper panel*) or total p38 (*lower panel*).

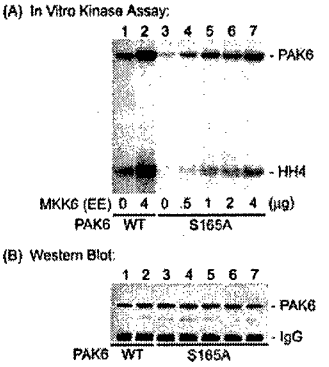

FIG. 3. **Substituting serine 165 with alanine down-regulates MKK6-induced PAK6 activation.** *A*, 293 cells were transiently co-transfected with HA-PAK6 WT or S165A and with various dosages of constitutively active mutant MKK6(EE). *In vitro* kinase activities were determined by a kinase assay on the immunoprecipitated PAK6. *B*, anti-HA Western blot of PAK6 WT and S165A mutants in *A*.

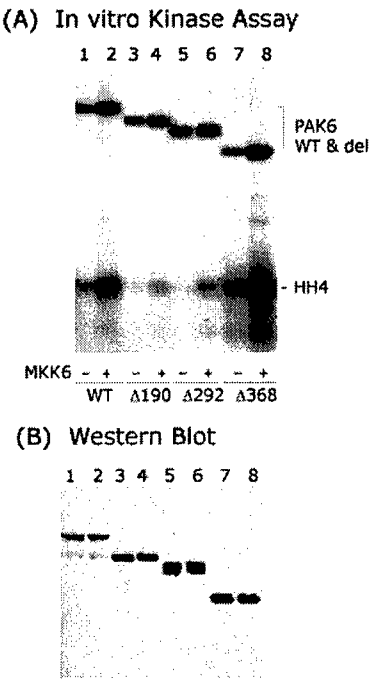

FIG. 4. **Mapping PAK6 domain susceptible to MKK6-induced activation.** *A*, 293 cells were co-transfected with MKK6(EE) and HA-tagged PAK6 WT full length and N-terminal deletion mutants Δ190, Δ292, or Δ368, respectively, for 24 h (all with N-terminal HA tags). The kinase activities of PAK6 WT, and N-terminal deletion mutants were determined by an IP kinase assay using anti-HA 12CA5 mAb. *B*, Western blot analysis showed equal levels of expression of PAK6 full length and deletions.

rylated by p38 MAP kinase. Aided by an on-line kinase substrate site analysis, Scansite (scansite.mit.edu), we identified a potential p38 MAP kinase phosphorylation site at serine 165 of PAK6. To characterize the role of Ser-165 in p38 MAP kinase-mediated PAK6 activation, we generated a serine-to-alanine (S165A) substitution mutant of PAK6 by site-directed mutagenesis. Consistent with SB203580 down-regulation of PAK6 kinase activity, substitution of Ser-165 with alanine dramatically reduced basal PAK6 kinase activity (Fig. 3*A*, *lane 1 versus lane 3*) as well as MKK6-stimulated activity (Fig. 3*A*, *lane 2 versus lane 7*). However, despite the down-regulation of kinase activity in the PAK6 S165A mutant, it remained responsive to MKK6-induced activation in a dose-dependent manner (Fig. 3*A*). Fig. 3*B* shows that the serine-to-alanine mutation, or MKK6 co-transfection, did not markedly alter PAK6 protein expression. Taken together, these findings indicated PAK6 could be activated by p38 MAP kinase-mediated phosphorylation of serine 165 but also suggested a second mechanism for activation by MKK6.

PAK6 Activity in Response to MKK6 Is Regulated by Sites in the Kinase Domain—The result that the S165A PAK6 mutant remained responsive to MKK6 indicated that additional target site(s) in PAK6 might be involved in p38 MAP kinase-mediated activation. To test this hypothesis, a series of PAK6 deletion mutants were employed to map additional region(s) of PAK6 that may participate in the MKK6-p38 MAP kinase-induced activation. Although the basal activities varied, co-transfected MKK6(EE) remained effective in up-regulating the kinase activities of PAK6 deletion mutants that extend from the N terminus to the region covering only the catalytic domain (Δ368 deletion mutant) (Fig. 4*A*). The levels of expression of wild type and deletion mutants in the control and MKK6(EE) co-transfected groups were similar, as shown in Fig. 4*B*. This result suggested that additional sites in the kinase domain were susceptible to activation by MKK6.

PAK6 Is Phosphorylated on Tyrosine upon MKK6 Activation—To identify potential MKK6-p38 MAP kinase target residue in the PAK6 kinase domain, we examined the sequence of PAK6 within this domain. No additional candidate p38 MAP kinase sites were found, but the activation loop contained a threonine-proline-tyrosine (TPY) sequence (residues 564–566) that resembled the substrate motif (TXY) recognized by MKK6 (14, 15). This motif was also present in the activation loop of MAP kinases and PAK1–6 but not in most other kinases (see Fig. 8*A*). MKK6 is a dual specificity kinase that recognizes and phosphorylates both threonine and tyrosine residues on the TXY motif of its substrate. The identification of TPY within the PAK6 activation loop suggested that PAK6 might be a direct substrate of MKK6. If this is the case, then one should detect increased tyrosine phosphorylation of PAK6 upon MKK6(EE)-induced activation. Reciprocal immunoprecipitation (IP)/Western blot analyses were performed to test this possibility.

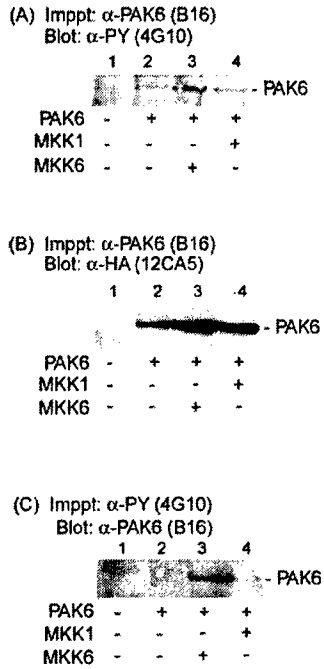

FIG. 5. **Tyrosine phosphorylation of PAK6 by MKK6 but not by MKK1.** 293 cells were co-transfected with HA-PAK6 and MKK6(EE) or MKK1(DD) and further incubated for 24 h. PAK6 was immunoprecipitated with anti-PAK6(B16) antiserum or with anti-phosphotyrosine (*α-PY*) 4G10 mAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal Western blot analysis. In both *A* and *C*, an increased tyrosine phosphorylation of PAK6 was evident in MKK6(EE) co-transfected group. *B*, comparable levels of PAK6 in the anti-PAK6(B16) immunoprecipitates were demonstrated by Western blot using anti-HA mAb.

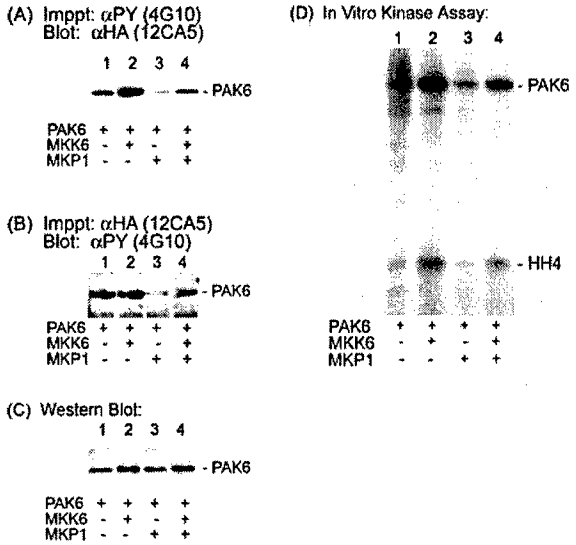

FIG. 6. **Down-regulation of MKK6-induced PAK6 tyrosine phosphorylation and kinase activation by dual specificity phosphatase MKP-1.** 293 cells were co-transfected with HA-PAK6 and MKK6(EE) or MKP-1 and further incubated for 24 h. PAK6 was immunoprecipitated with anti-HA 12CA5 mAb or with anti-phosphotyrosine (*αPY*) 4G10 mAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal Western blot analysis. The results are shown in *A* and *B*. MKP-1 dephosphorylates PAK6 tyrosine residue under basal and MKK6-stimulated conditions, as evident from the reduced immunoreactivity of PAK6 with anti-phosphotyrosine 4G10 mAb. *C*, comparable amounts of PAK6 in lysates derived from each group were demonstrated by Western blot analysis. *D*, down-regulation of PAK6 kinase activity by MKP-1 co-transfection was demonstrated by an IP kinase assay using 293 cells co-transfected with PAK6 and with MKK6(EE) or MKP-1. Kinase activity was determined by an *in vitro* kinase assay with [^{32}P]ATP using the anti-HA immunoprecipitated kinase and analyzed by SDS-PAGE followed by autoradiography.

PAK6 was immunoprecipitated from transfected cells with a polyclonal antibody raised against PAK6 (B16) and then immunoblotted with anti-phosphotyrosine antibody (4G10). An increase in tyrosine phosphorylation in the anti-PAK6 immunoprecipitates was detected when PAK6 was co-expressed with MKK6(EE) (Fig. 5*A*). In contrast, no change in tyrosine phosphorylation of PAK6 was detected when it was co-transfected with the constitutively active MKK1(DD), a related member of the MAP kinase kinase family (16). Immunoblotting with anti-HA showed that the immunoprecipitates contained comparable levels of total PAK6 (Fig. 5*B*). In the reciprocal experiment, the lysates were immunoprecipitated with the anti-phosphotyrosine 4G10 antibody and then immunoblotted for PAK6. As shown in Fig. 5*C*, MKK6 co-transfection increased the level of PAK6 that was immunoprecipitated by anti-phosphotyrosine 4G10.

To further address the phosphorylation of Tyr-566 by MKK6, a dual specificity phosphatase, MKP-1, which can dephosphorylate both threonine and tyrosine on the TXY motif (17), was tested in IP/Western blot experiments. MKP-1 co-transfection in the absence of MKK6(EE) markedly decreased the basal tyrosine phosphorylation of PAK6 (Fig. 6, *A* and *B*, *lane 1 versus lane 3*). MKP-1 co-transfection similarly reduced the level of MKK6-induced tyrosine phosphorylation of PAK6 (Fig. 6, *A* and *B*, *lane 2 versus lane 4*). Fig. 6*C* demonstrates that total PAK6 expression levels were not altered by MKP-1 (*lane 1 versus lane 3* and *lane 2 versus lane 4*). Finally, *in vitro* kinase assays were carried out to assess the correlation between the loss of tyrosine phosphorylation induced by MKP-1 and PAK6 kinase activity. Consistent with the tyrosine phosphorylation results, MKP-1 down-regulated basal and MKK6-stimulated PAK6 kinase activity on the exogenous histone H4 substrate (Fig. 6*D*). Basal autophosphorylation was also decreased by MKP-1, although the effects on autophosphorylation in the MKK6-transfected cells were less prominent.

Substitution of Tyr-566 with Phenylalanine Down-regulates PAK6 Activation by MKK6—To assess more directly the involvement of Tyr-566 phosphorylation in PAK6 activation, we generated a mutant PAK6 (Y566F) by substituting the Tyr-566 residue with a phenylalanine. This mutation markedly reduced the level of basal PAK6 tyrosine phosphorylation (Fig. 7, *A* and *B*, *lane 1 versus lane 3*). Similarly, the Y566F mutation markedly reduced the tyrosine phosphorylation stimulated by MKK6 (Fig. 7, *A* and *B*, *lane 2 versus lane 4*). Fig. 7*C* demonstrates that the wild type and mutant PAK6 constructs were expressed at comparable levels. These results indicated that Tyr-566 was a major site of basal and MKK6-stimulated tyrosine phosphorylation, although perhaps not the only site, because the Y566F mutation did not completely eliminate MKK6-induced tyrosine phosphorylation.

We next examined the effects of the Y566F mutation on basal and MKK6-stimulated PAK6 kinase activity. Substitution of Tyr-566 with phenylalanine reduced basal PAK6 autophosphorylation and kinase activity toward the exogenous histone H4 substrate (Fig. 7*D*, *lane 1 versus lane 3*) and reduced the magnitude of MKK6-stimulated PAK6 kinase activation (Fig. 7*D*, *lane 2 versus lane 4*). Moreover, a double mutation of S165A and Y566F completely abrogated the MKK6-stimulated PAK6 activation (Fig. 7*D*, *lane 8*). Therefore, although MKK6 may directly or indirectly stimulate the phosphorylation of additional tyrosines, serine 165 and tyrosine 566 appear to be the critical sites mediating MKK6-stimulated PAK6 kinase activity. Taken together, these data indicate that MKK6 activates PAK6 by direct phosphorylation of the TXY motif located

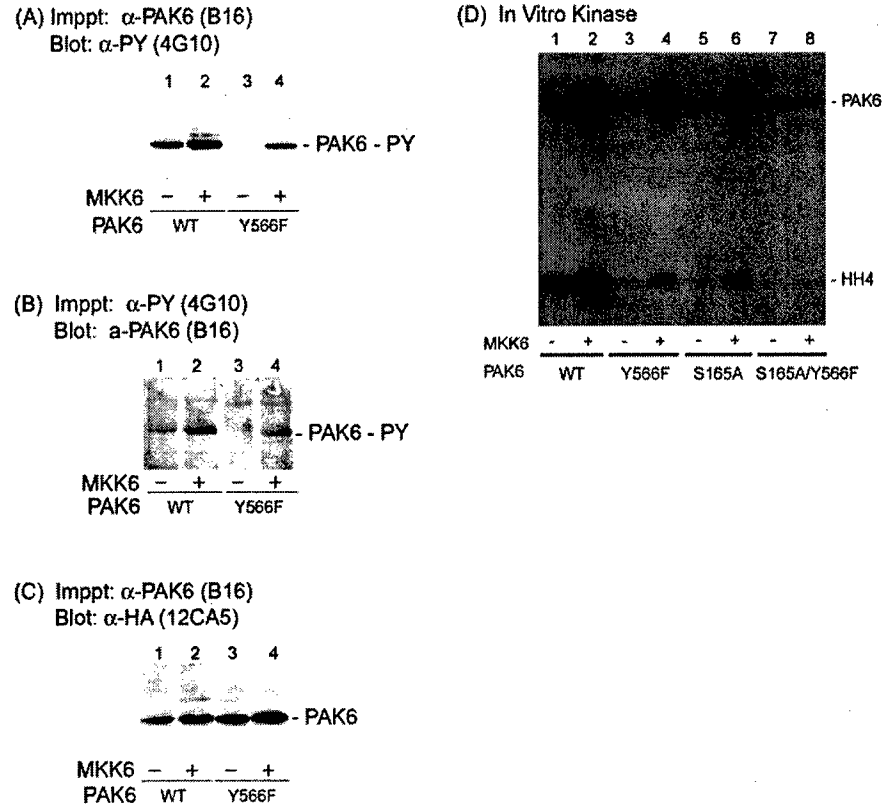

FIG. 7. **Substitution of Tyr-566 with phenylalanine (Y566F) dampens MKK6-stimulated PAK6 activation.** Levels of tyrosine phosphorylation were evaluated between PAK6 WT and Y566F mutant by IP/Western blot analysis using 293 cells transiently expressing MKK6-(EE) and PAK6 (WT or Y566F). PAK6 was immunoprecipitated with anti-HA 12CA5 mAb or with anti-phosphotyrosine (*α-PY*) 4G10 mAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal Western blot. The results are shown in *A* and *B*. Reduced levels of tyrosine phosphorylation of PAK6 in basal and MKK6-stimuated conditions were evident in the Y566F group. *C*, Western blot demonstrated the comparable amount of PAK6 in anti-HA immunoprecipitates. *D, in vitro* IP Kinase assay demonstrated down-regulation of PAK6 kinase activity by substituting tyrosine 566 with phenylalanine (Y566F) at both basal nonstimulated and MKK6-stimulated conditions (*lanes 3* and *4*). Double mutation of serine 165 and tyrosine 566 (S165A/Y566F) abrogated MKK6-induced PAK6 activation (*lanes 7* and *8*).

within the activation loop and by stimulating p38 MAP kinase-mediated phosphorylation of serine 165.

MKK6/p38 MAP Kinase Pathway Regulates Other Members of PAK Family—As indicated above, the activation segments of PAK1–6 all contain similarly positioned TPY sequences (Fig. 8*A*). These are located four amino acids C-terminal to conserved serines (PAK4–6) or threonines (PAK1–3), which are autophosphorylated and also regulate kinase activity (see below). This TXY motif is absent from most other serine/threonine kinases, and the positioning of the motif in the MAP kinases is distinct. These observations suggest that activation induced by MKK6 may be common among PAK family kinases. Therefore, additional members of the PAK family (HA-tagged PAK1, PAK4, and PAK5) were tested in the same co-transfection studies coupled with *in vitro* kinase assays.

Consistent with previous reports, PAK4 and PAK5 had readily detectable basal kinase activities. Both PAK4 (Fig. 8*B*, *lanes 5* and *6*) and PAK5 (Fig. 8*B*, *lanes 3* and *4*) responded to MKK6(EE) co-transfection with, respectively, 4.7- and 3.2-fold increases of kinase activity in a fashion similar to PAK6 (Fig. 8*D*, *lower panel*). In contrast, PAK1 was inactive both in the absence and presence of MKK6(EE) (Fig. 8, *B*, *lanes 7* and *8*, and *D*, *lower panel*) but was strongly stimulated by co-transfection with a constitutively active valine 12 mutant of Cdc42 (Cdc42-V12). However, MKK6(EE) co-transfection had a marginal effect on further promoting Cdc42-activated PAK1 activity, with no change in enzymatic activity toward exogenous substrate histone H4 and a small 22% increase of autophosphorylation (Fig. 8, *B*, *lanes 9* and *10*, and *D*). Immunoblotting with an anti-HA antibody confirmed that each of the kinases was expressed at comparable levels within each group (Fig. 8*C*). These results indicate that members of the group 2 PAK family (PAK4, 5, and 6) share a common mechanism of being stimulated via the MKK6-p38 MAP kinase pathway.

Serine 560 Phosphorylation in Activation Loop Is Required for MKK6-mediated Stimulation—A serine residue conserved among group 2 PAKs is located at position 560 within the activation loop of the PAK6 kinase domain (Fig. 8*A*). This serine residue corresponds to the autophosphorylated regulatory threonine 423 in the activation loop of PAK1 and was previously shown (using a phosphoserine 560-specific antibody) to be autophosphorylated under basal conditions in PAK6 (11). This phospho-specific antibody was used to determine whether MKK6-p38 MAP kinase activation increased phosphorylation at this site. As shown in Fig. 9*A*, MKK6 co-transfection did not alter Ser-560 phosphorylation level (*lane 1 versus lane 2*). The specificity of the antibody was confirmed by the lack of reactivity to mutants in codon 560 (*lanes 3–5*), and equivalent total PAK6 expression was confirmed by immunoblotting for the HA epitope tag (Fig. 9*B*). Finally, although MKK6 did not increase Ser-560 phosphorylation, we next determined whether phosphorylation at this site was necessary for PAK6 activation by MKK6-p38 MAP kinase. Significantly, mutation of this site to alanine (S560A) markedly diminished the basal activity and abrogated stimulation by MKK6 (Fig. 9*C*, *lanes 3* and *4*).

In PAK1, substitution of threonine 423 with a glutamic acid (T423E) that mimics a negatively charged phosphothreonine residue results in constitutive activation of the kinase, whereas substituting threonine with alanine ablates kinase activity (12, 13). To further characterize the role of serine 560 phosphorylation in PAK6 activation, we generated PAK6 mutants by substituting the serine 560 with either negatively charged glutamic acid (S560E) or aspartic acid (S560D). The enzymatic activities of these mutants were tested along with wild type PAK6 and MKK6-p38 MAP kinase activated wild type PAK6. As shown in Fig. 9*C* (*lanes 5* and *7*), substitution of negatively charged residues (S560E or S560D) did not stimulate kinase activity but instead markedly repressed basal kinase activity. Similarly to the S560A mutation, these mutations also completely abrogated PAK6 activation by MKK6 (*lanes 6* and *8*). Taken together, these results indicated a critical MKK6-inde-

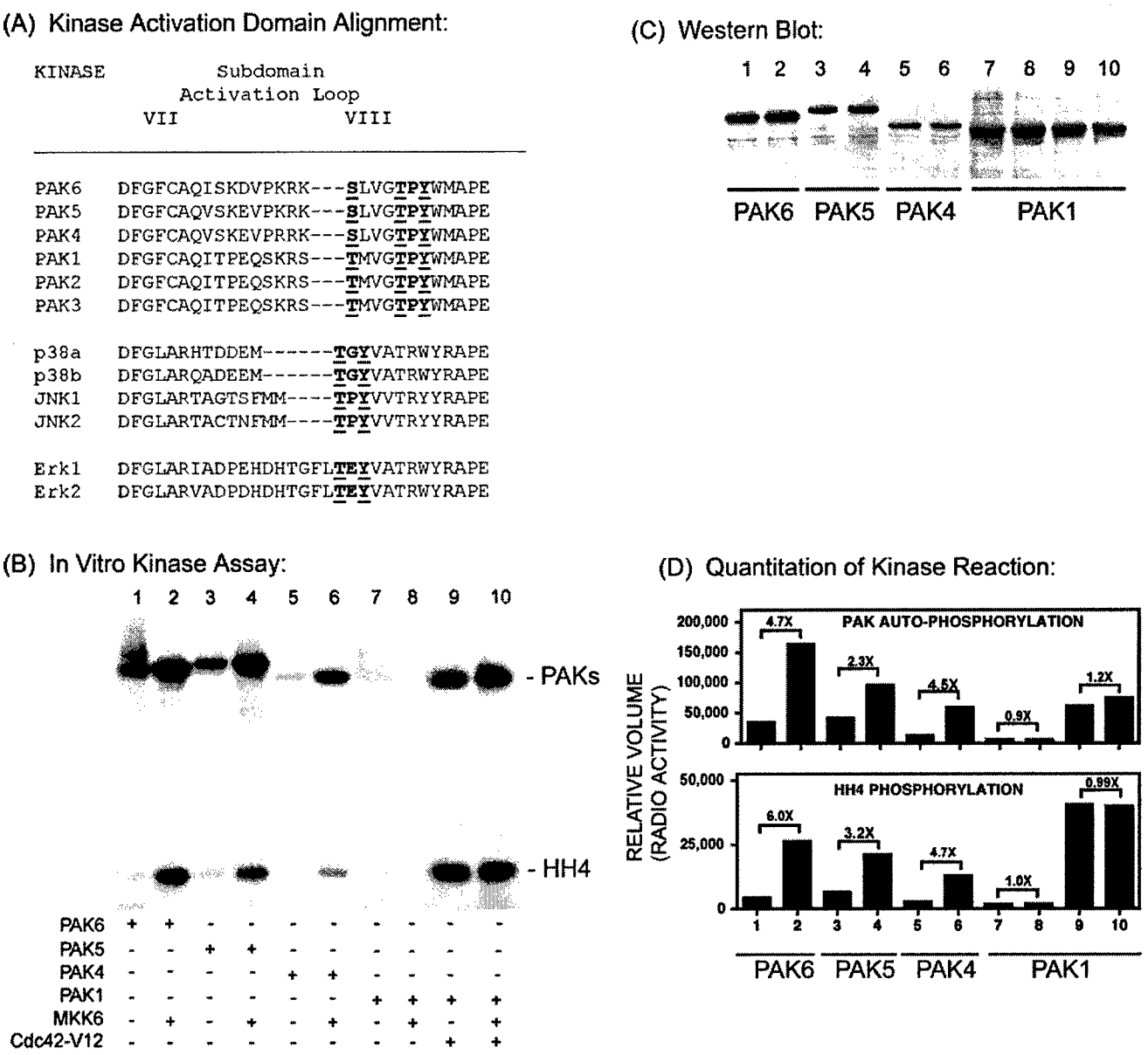

FIG. 8. **Specificity of MKK6-induced PAK activation.** *A*, sequence alignment of the activation loop region between catalytic subdomains VII and VIII of PAKs and MAP kinase family members. In both group 1 and group 2 PAKs, in addition to the previously defined autophosphorylated threonine or serine residues, a conserved TXY motif was also found within the activation loop. *B*, kinase activity of MKK6-modulated PAKs. 293 cells were co-transfected with MKK6(EE) and one of the following HA-tagged PAK family members: PAK1, PAK4, PAK5, or PAK6. Additionally, PAK1 was also tested by co-transfecting with a constitutive active Cdc42-V12. The kinase activity was determined by an *in vitro* kinase assay with anti-HA mAb immunoprecipitated kinases and analyzed by SDS-PAGE followed by autoradiography. *C*, Western blot demonstrated the comparable level of expression within individual PAK testing group. *D*, quantitation of kinase reactions from *B* using a PhosphorImager. The *upper panel* depicts levels of autophosphorylation of various PAKs. The *lower panel* depicts kinase activity toward histone H4. The *numbers* on *top* of each PAK group indicate fold activation in response to MKK6 co-transfection.

pendent role for serine 560 autophosphorylation in regulating PAK6 kinase activity.

DISCUSSION

PAK6 is classified as a PAK family member based on homology in the kinase domain and in its N-terminal CRIB domain. However, in contrast to PAK1 and the other group 1 PAKs, PAK6 kinase activity is not stimulated by Cdc42 or Rac binding, and the mechanisms that regulate its kinase activity have been unclear. This study found that basal PAK6 kinase activity was repressed by a p38 MAP kinase antagonist and could be strongly stimulated by activation of the MKK6-p38 MAP kinase pathway. A role for p38 MAP kinase in directly regulating PAK6 was further supported by a marked decrease in kinase activity upon mutation of a consensus target site at serine 165. PAK6 was also directly activated by MKK6, and this activation was dependent upon tyrosine 566 in the activation loop of the PAK6 kinase domain. Significantly, this tyrosine is part of an MKK6 dual specificity kinase substrate motif, TXY, that is found in the activation loop of MAP kinases and in the other PAKs but is absent from most other kinases. Finally, PAK6 kinase activity was also dependent upon an autophosphorylated serine (serine 560) in the activation loop. These results indicate that PAK6 kinase activity is regulated by both autophosphorylation and MAP kinase kinase-mediated phosphorylation of residues in its activation loop and by p38 MAP kinase mediated phosphorylation outside the kinase domain.

The kinase activity of PAK1 is regulated by an AID in the

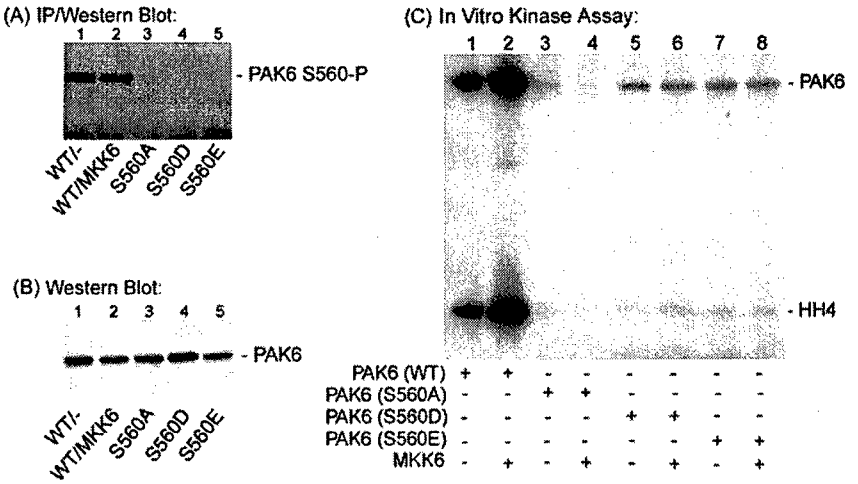

FIG. 9. **PAK6 does not respond to MKK6-induced activation with increased Ser-560 phosphorylation.** *A*, 293 cells transiently co-transfected with HA-tagged PAK6 Ser-560 substitution mutants, serine to alanine (S560A), serine to aspartic acid (S560D), or serine to glutamic acid (S560E), were immunoprecipitated by anti-HA. 12CA5 monoclonal antibody was subjected to Western blot with an anti-Ser-560 phospho-specific antibody. *B*, anti-HA Western blot showed comparable amount of PAK6 protein expressed in the each testing group. *C*, PAK6 Ser-560 substitution mutants S560A, S560D, and S560E along with wild type were transfected without or with MKK6(EE), and the resulting kinase activity was analyzed by IP/kinase assays.

N-terminal half of the molecule downstream of the CRIB domain, which binds to and represses the catalytic domain (1, 2, 12, 18–21). The binding of GTP-Rac or GTP-Cdc42 to the CRIB domain causes the AID to dissociate from the catalytic domain, with subsequent phosphorylation of residues in the AID and of Thr-423 in the activation loop of the catalytic domain (12, 13, 18, 22). In contrast to PAK1 and the other group 1 PAKs (PAK2 and PAK3), this AID is not conserved in PAK6 or in the other group 2 PAKs (PAK4 and PAK5), and the kinase activities of the group 2 PAKs are not stimulated by Cdc42 binding. Nonetheless, the group 2 PAKs contain serine at a position homologous to the autophosphorylated threonine 423 of PAK1 (serine 560 in PAK6). Significantly, constitutive autophosphorylation of this serine 560 in PAK6 was recently demonstrated using a phospho-specific anti-PAK6-Ser-560 antibody (11), consistent with the lack of an AID.

Thr-423 in PAK1 and Ser-560 in PAK6 are located at the center of the activation loop within the catalytic domain. Crystallographic studies of PAK1 indicate that the phosphorylation of this residue stabilizes the interaction between the activation loop and substrate (1). Consequently, substituting Thr-423 with a negatively charged glutamic acid residue renders PAK1 constitutively active. In PAK4, mutating the corresponding Ser-473 to glutamic acid also results in constitutive kinase activity (23, 24). In contrast, we found that substituting the PAK6 Ser-560 with glutamic acid or aspartic acid diminished kinase activity, indicating that these negatively charged residues were not equivalent to phosphorylation in the case of PAK6. More importantly, replacement of serine 560 with alanine resulted in the almost complete abrogation of kinase activity, confirming a critical role for this site.

Although PAK6 does not appear to have an AID, and Ser-560 phosphorylation appears to be constitutive and required for kinase activity, we cannot yet rule out the possibility that Ser-560 phosphorylation also negatively regulates PAK6 interaction with an undefined inhibitory domain. One recent report describes as the "autoinhibitory" domain of the PAK5 a region that bears almost no sequence homology to other members of the PAK family (25). This region (residues 60–180) is located directly downstream of the PAK5 CRIB domain and appears to be able to down-regulate PAK5 kinase activity *in vitro*. This result raises the interesting possibility that regions immediately following the CRIB domain of group 2 family PAKs may regulate the catalytic domain. If this is the case for PAK6, then the identification of serine 165 as a p38 MAP kinase substrate site would suggest that PAK6 activation may be initiated by phosphorylation of this site and that this activation signal may then be amplified by Ser-560 phosphorylation and subsequent MKK6 phosphorylation of the activation loop.

The highly conserved activation loops in PAK1–6, each containing the TXY motif, indicate that dual regulation by autophosphorylation and MKK6 may be common to other members of the PAK family. Indeed, the kinase activities of PAK4 and PAK5 were also stimulated by MKK6, consistent with the MKK6 regulation of the group 2 PAKs. In contrast, in response to MKK6 co-transfection, only a marginal increase of Cdc42-activated PAK1 autophosphorylation was observed, but its kinase activity toward exogenous substrate histone H4 remained unchanged. This was not due to a dominant inhibitory effect of the AID, because MKK6 failed to enhance the activation mediated by activated Cdc42. These results suggest that activation by MKK6 may be unique among the group 2 PAKs, although it is possible that there are more subtle effects on PAK1 or that additional priming events are needed. Alternatively, the TPY motif in the group 1 PAKs may be recognized by another kinase.

Although the PAK and MAP kinase families appear to share a functional TXY MAP kinase kinase motif, its position is shifted toward the C terminus of the activation loop in the PAKs. Moreover, PAKs differ from the MAP kinases in that they have a regulatory serine or threonine located in the center of the activation loop. Further structural studies are needed to determine precisely how phosphorylation at these multiple sites affects the activation loop and kinase activity. Nonetheless, the shared mechanism of activation by PAK6 and p38 MAP kinase suggests that PAK6 (and likely other PAKs) has a unique and specialized role in the cellular response to stress-related signals.

Acknowledgment—We thank C. Sylvia Lin for editorial assistance in the preparation of the manuscript.

REFERENCES

1. Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000) *Cell* **102,** 387–397
2. Parrini, M. C., Lei, M., Harrison, S. C., and Mayer, B. J. (2002) *Mol. Cell* **9,** 73–83
3. Lee, S. R., Ramos, S. M., Ko, A., Masiello, D., Swanson, K. D., Lu, M. L., and Balk, S. P. (2002) *Mol. Endocrinol.* **16,** 85–99
4. Yang, F., Li, X., Sharma, M., Zarnegar, M., Lim, B., and Sun, Z. (2001) *J. Biol. Chem.* **276,** 15345–15353
5. Jaffer, Z. M., and Chernoff, J. (2002) *Int. J. Biochem. Cell Biol.* **34,** 713–717
6. Abo, A., Qu, J., Cammarano, M. S., Dan, C., Fritsch, A., Baud, V., Belisle, B., and Minden, A. (1998) *EMBO J.* **17,** 6527–6540
7. Dan, C., Kelly, A., Bernard, O., and Minden, A. (2001) *J. Biol. Chem.* **276,** 32115–32121
8. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) *Mol. Cell. Biol.* **16,** 1247–1255
9. Han, J., Lee, J. D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R. J. (1996) *J. Biol. Chem.* **271,** 2886–2891

10. Haq, R., Brenton, J. D., Takahashi, M., Finan, D., Finkielsztein, A., Damaraju, S., Rottapel, R., and Zanke, B. (2002) *Cancer Res.* **62,** 5076–5082
11. Schrantz, N., da Silva Correia, J., Fowler, B., Ge, Q., Sun, Z., and Bokoch, G. M. (2004) *J. Biol. Chem.* **279,** 1922–1931
12. Zenke, F. T., King, C. C., Bohl, B. P., and Bokoch, G. M. (1999) *J. Biol. Chem.* **274,** 32565–32573
13. King, C. C., Gardiner, E. M., Zenke, F. T., Bohl, B. P., Newton, A. C., Hemmings, B. A., and Bokoch, G. M. (2000) *J. Biol. Chem.* **275,** 41201–41209
14. Jiang, Y., Li, Z., Schwarz, E. M., Lin, A., Guan, K., Ulevitch, R. J., and Han, J. (1997) *J. Biol. Chem.* **272,** 11096–11102
15. Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R. J., Luo, Y., and Han, J. (2002) *Science* **295,** 1291–1294
16. Milanini, J., Vinals, F., Pouyssegur, J., and Pages, G. (1998) *J. Biol. Chem.* **273,** 18165–18172
17. Clark, A. R. (2003) *J. Endocrinol.* **178,** 5–12
18. Chong, C., Tan, L., Lim, L., and Manser, E. (2001) *J. Biol. Chem.* **276,** 17347–17353
19. Buchwald, G., Hostinova, E., Rudolph, M. G., Kraemer, A., Sickmann, A., Meyer, H. E., Scheffzek, K., and Wittinghofer, A. (2001) *Mol. Cell. Biol.* **21,** 5179–5189
20. Thompson, G., Owen, D., Chalk, P. A., and Lowe, P. N. (1998) *Biochemistry* **37,** 7885–7891
21. Osada, S., Izawa, M., Koyama, T., Hirai, S., and Ohno, S. (1997) *FEBS Lett.* **404,** 227–233
22. Renkema, G. H., Pulkkinen, K., and Saksela, K. (2002) *Mol. Cell. Biol.* **22,** 6719–6725
23. Qu, J., Cammarano, M. S., Shi, Q., Ha, K. C., de Lanerolle, P., and Minden, A. (2001) *Mol. Cell. Biol.* **21,** 3523–3533
24. Callow, M. G., Clairvoyant, F., Zhu, S., Schryver, B., Whyte, D. B., Bischoff, J. R., Jallal, B., and Smeal, T. (2002) *J. Biol. Chem.* **277,** 550–558
25. Ching, Y. P., Leong, V. Y., Wong, C. M., and Kung, H. F. (2003) *J. Biol. Chem.* **278,** 33621–33624

Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer

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Running Title: Rafts and nongenomic signaling

Supported by NIH R37 DK47556, R01 DK57691, and P50 DK65298 (to M.R.F) and R01 CA87997 and DoD DAMD17-02-0017 (to M.L.L.). B.C. is the recipient of a DoD Postdoctoral Traineeship Award (PC030618). Children's Hospital/Harvard Urological Diseases Research Center is an NIDDK-sponsored George M. O'Brien Center.

Abbreviations

AR, androgen receptor
CSD, caveolin scaffolding domain
E2, 17β-estradiol
eNOS, endothelial nitric oxide synthase
EGFR, epidermal growth factor receptor
ERα/β, estrogen receptor α,β
ERK, extracellular signal regulated protein kinase
hCG, human chorionic gonadotrophin
IGF-1R, insulin-like growth factor-1 receptor
IL-4, -6, interleukin-4 and -6
LBD, ligand binding domain
MAP kinase, mitogen activated protein kinase
MNAR, modulator of nongenomic activity of estrogen receptor
mTOR, mammalian target of rapamycin
PCa, prostate cancer
PI3K, phosphoinositide-3-kinase
PKB, protein kinase B (Akt)
PR, progesterone receptor
pTyr, phosphotyrosine
SH2/SH3, Scr-homology domain 2 or 3
STAT3, signal transducer and activator of transcription-3

Abstract

Emerging evidence indicates that nuclear receptors for steroid hormones can signal by "nongenomic" mechanisms that operate independently of their transcription function. These signal transduction processes occur within seconds to minutes after initiation with agonist and involve interactions of the nuclear receptors with other signaling proteins in the cytoplasm and at membrane surfaces. This review provides an overview of published information on possible nongenomic activities of the androgen receptor (AR) and other nuclear receptors, with a focus on the potential involvement of these processes in prostate cancer. We discuss the hypothesis that the cholesterol-rich lipid raft compartment(s) of cancer cell membranes may provide privileged sites for nongenomic signals mediated by the AR.

Androgen and prostate cancer

Prostate cancer (PCa) is a leading cause of premature death in Western countries [1]. An estimated 189,000 men were diagnosed with PCa in 2002 in the US and in the same year an estimated 30,200 died from this disease [2]. There is no effective therapy for disseminated PCa. Late-stage patients are managed primarily in a palliative manner and experience considerable morbidity. Androgen ablation remains, after many decades, the principal treatment paradigm for PCa not confined to the primary site. However, relapse from hormonal therapy is almost certain and most patients with metastases progress to end-stage disease regardless of treatment strategy. The lack of progress on clinical options for PCa patients reflects our poor understanding of the molecular and cellular mechanisms underlying disease etiology and progression.

PCa is known as a "hormone-dependent" disease because the prostate requires testicular androgens for the performance of its secretory function, and PCa cells retain this sensitivity to androgen. Androgens are believed to be the primary soluble mediators of growth and homeostatic survival of prostate epithelial cells *in situ* [3]. PCa cell growth and survival pathways respond decisively to androgen, indicating that this class of steroids is a fundamental mediator of PCa cell physiology. An extensive literature exists on this topic and most investigators believe that androgenic signaling is important for tumor growth and disease progression in prostatic maligancy. Nevertheless, aggressive PCa is generally considered to be "androgen-<u>in</u>dependent" because of the uniform failure of therapies designed to block androgenic signaling. The mechanisms by which PCa progresses from an androgen-dependent to an androgen-independent disease are not understood.

The principal molecular target for androgen is the androgen receptor (AR), a member of the nuclear receptor family of ligand-dependent transcription factors. AR binds to specific chromatin regions in concert with other transcriptional regulators and controls

the expression of a wide range of target genes [4]. Although AR function is normally highly dependent on the availability of sufficient levels of androgen to directly bind and activate the receptor, many independent lines of evidence now indicate that the AR likely plays a major role in androgen-independent disease, i.e., in the absence of significant levels of hormone [5]. The mechanisms underlying AR activation by the extremely low levels of androgen present in the castrate condition, or the manner in which "ligand-independent" activation of the AR can occur, are only beginning to be revealed. The changes in androgen signaling that occur in PCa are diverse, and include alterations in the availability or activity of AR co-activators and repressors [4,6,7], somatic mutations in the AR gene that change the expression level, ligand-binding or other properties of the receptor [8-10], and changes in signaling mechanisms that impinge on AR-mediated signal transduction pathways [11,12]. Despite recent insights into the manner in which the AR contributes to disease progression, the precise roles played by the AR in PCa are still poorly understood, particularly in the low androgen condition.

Nongenomic nuclear receptor signals

In the classical paradigm, AR exerts its biological effects by activating transcription of target genes, a process known as the "genotropic signal". In addition to the well-established role of steroid hormone receptors as transcription factors, there is now considerable experimental evidence that AR [13-17], estrogen receptors -α and -β (ERα/ERβ) [13,18], several types of progesterone receptors (PR) [19-21], and other proteins in the nuclear receptor protein family [22], can mediate signaling activities by "nongenomic" mechanisms [23]. A nongenomic activity is understood to be extremely rapid (min vs h) and indifferent to treatment with inhibitors of transcription or protein synthesis, such as actinomycin D and cycloheximide. Evidence that steroids can exert physiological effects rapidly (e.g., effects of estrogen that occur in tissues within seconds) has actually existed for decades [24], however it was not until recently that some of the mechanistic aspects of this process came into view.

Although some nongenomic effects of nuclear receptor ligands may be resistant to nuclear receptor antagonists [25], it is now clear that nongenomic steroidal effects can involve the classical nuclear receptors [19]. This conclusion arises because rapid actions of steroids have been described that can be completely inhibited by well-characterized pharmacologic antagonists of the classic receptor proteins [13,18]. In addition, the fact that steroid receptors reside in multiprotein complexes in the cytoplasm prior to ligand binding and nuclear translocation allows for the possibility of productive interactions with molecules in the cytosol or at extranuclear membrane surfaces. Because activated, ligand-bound receptors require 30 min to 1 h to translocate to the nucleus, such interactions are also theoretically possible in the presence of hormone[26].

Cytosolic signal transduction cascades have been demonstrated to be rapidly activated by mechanisms involving classic steroid hormone receptors and cytoplasmic and membrane effector proteins. 17β-estradiol (E2) was shown to activate endothelial nitric oxide synthase (eNOS) in vascular endothelial cells within minutes by a mechanism involving ERα phosphoinositide-3-kinase (PI3K) and the Akt/PKB serine-threonine kinase [18]. This rapid process, which complements classical genomic effects of estrogen on vascular pathways [27], may partially explain the cardioprotective effects of estrogen. ERα but not ERβ, was found capable of activating signaling from the insulin-like growth factor-1 (IGF-1) receptor by a mechanism involving direct binding of ERα to the IGF-1R [28]. Membrane ERα has also been recently shown to form signaling complexes with other receptor tyrosine kinases, such as the proto-oncoprotein ErbB2, with subsequent activation of the PI3K-Akt pathway [29].

Although there is currently less information on possible nongenomic roles for the AR than for the ERs, nongenomic actions of androgen have been described in at least 10

different cell types [30]. Some of these may involve interactions between ligand and nonclassical receptor systems. Although only one AR has been identified to date, three isoforms of novel membrane progesterone receptors [20,21], a family of seven transmembrane G protein coupled receptors, were recently cloned and characterized, demonstrating that specialized, membrane-localized steroid receptors may be involved in the generation of certain types of rapid signals. Given the structural similarity between the ligand binding domains of classical AR and PR, it is plausible that novel membrane ARs belonging to this family may yet be found.

Perhaps the most unambiguous example of a distinctive nongenomic signal mediated by classical ARs is the recent demonstration that oocyte maturation, a process elicited by steroid hormones in seconds to minutes and which does not involve gene transcription, is susceptible to inhibition by classical AR antagonists in both Xenopus [16,17] and mouse [31] oocytes. Although progesterone was thought to be the physiologically relevant steroid involved in oocyte maturation in Xenopus, examination of ovarian extracts following injection of frogs with human chorionic gonadotrophin (hCG) revealed nearly undetectable levels of progesterone, while androstenedione and testosterone were abundant[16]. Thus, studies of steroid effects on oocytes suggest that androgens may be the primary physiologic mediator of release from meiotic arrest in oocyte maturation, with the principal hormonal effect being activation of a nongenomic, rather than a classical genotropic, signaling mechanism. Although the oocyte system is a rather specialized case, these observations demonstrate the potential for physiologically important membrane- or cytoplasm-initiated androgenic signals.

Another recently reported nongenomic pathway was demonstrated in experiments with the LNCaP human PCa cell line. Cells treated with E2 or androgen resulted in stimulation of cell proliferation mediated by the rapid formation of a cytosolic signaling

complex containing ERα or ERβ (depending on cell type), AR and the nonreceptor tyrosine kinase, Src [13]. In that study, complex formation and downstream signaling were inhibited by specific ER and AR antagonists, verifying the participation of the classical steroid receptors in the complex. Interaction between the SH2 domain of Src was mapped to the pTyr 537 residue of ERα and interaction with the Src SH3 domain with a proline-rich region of the AR. More recently, a scaffolding protein named "modulator of nongenomic activity of estrogen receptor" (MNAR) was identified as an element of this signaling pathway [32]. Upon E2 stimulation, a ternary complex comprised of ERα, Src and MNAR is formed and activation of Src/MAP kinases ensues. MNAR contains multiple LXXLL motifs as well as two proline-rich regions with multiple PXXP motifs, which mediate interactions with ERα and the Src SH3 domain, respectively. Further clarification of the rates of formation and the protein components of this and similar cytosolic complexes activated by hormones will provide additional insight into the manner in which extranuclear steroid receptors are involved in processing signals relevant to tumor cell behavior.

Targeting AR to lipid rafts/caveolae

Despite accumulating evidence of signaling events involving cytosolic, membrane-proximal AR, the mechanism of AR targeting to, and participation in, signaling at or near the plasma membrane or other cellular membranes is still unclear. One potential mechanism is direct association of AR with an integral membrane component through direct interaction. In support of this notion, AR was recently shown by one of us (M.L.L.) to reside in LNCaP cells in association with low density membrane fractions isolated by sucrose gradient ultracentrifugation [33]. In that study, AR was demonstrated to interact directly with the integral membrane protein, caveolin-1. Using a mammalian two-hybrid system, AR domains required for interaction with caveolin included both the N-terminal AF1 domain and the ligand-binding domain (LBD). This study on AR is mirrored by the finding that ERα can also specifically interact with caveolin-1 [34]. Interaction motifs within ERα were mapped

to the N-terminal AF1 domain; specifically, to two motifs, YNYPEGAAY and FGSNGLGGF, which were shown to mediate the interaction with the caveolin scaffolding domain (CSD). The CSD was previously identified in caveolin-interacting molecules that contain either ϕXXϕXXXXϕ or ϕXXXXϕXϕ (ϕ represents a hydrophobic residue and X can be any residue). Peptides containing this motif have been shown to disrupt interactions with caveolin. Interestingly, similar CSD binding motifs are also present in AR (YSWMGLMVFAMGWRSF) and in mineralocorticoid receptor (FPFMDGSYFSF), although the potential role of these regions in signaling remains to be characterized.

A recent study by Acconcia et al. [35] demonstrated that palmitoylation of cysteine 477 in the LBD of ERα mediates its plasma membrane localization. HeLa cells ectopically expressing a Cys477Ala substitution mutant failed to localize to ERα to the plasma membrane. Significantly, ERα Cys477Ala failed to elicit E2-induced rapid activation of ERK/MAP kinase signaling. E2 was also shown to reduce both ERα palmitoylation and its interaction with caveolin in a time- and dose-dependent manner. Although AR has not been shown to be palmitoylated, the findings with ERα underscore the relevance of posttranslational modifications with membrane targeting capacity to hormone receptor function. These modifications may provide the opportunity for permissive or facilitated interactions between the nuclear receptor proteins and integral membrane proteins, such as caveolins, with implications for regulation of downstream signaling.

Cytosolic androgen receptor and cholesterol-rich lipid rafts

The links between AR and floating fractions in sucrose gradients and to caveolin-1 described above place AR within plasma membrane structures called caveolae and/or within cytosolic membrane organelles that communicate with caveolae. Caveolae are an invaginated form of membrane microdomain most commonly referred to as "lipid rafts". Lipid rafts are cholesterol- and sphingolipid-rich components of the plasma membrane that

are resistant to extraction with cold nonionic detergents and which exhibit light buoyant density in sucrose gradients [36]. Because of their lipid composition, detergent-resistant, lipid raft membranes, which probably comprise about 10% of the plasma membrane area, possess a "liquid-ordered" structure that distinguishes these regions from the majority, liquid-disordered fraction of the plasma membrane. A large amount of evidence supports the view that lipid rafts are discrete membrane domains that exist in living cells and that perform multiple physiologic functions [37-47]. However, difficulties in definition, measurement of their size, and in the meaning of "detergent-insolubility" have generated controversies in the field that have not yet been resolved [36,48-50]. Caveolae, which exhibit an invaginated architecture identifiable unambiguously in electron micrographs, were discovered in the 1950s and are the best-studied form of cholesterol-rich raft [40]. The invaginated structure of caveolae results from the presence within these membrane compartments of one or more members of the caveolin family (caveolin-1, -2, and –3) [51]. Caveolins possess an unusual transmembrane structure and bind cholesterol and many cell signaling proteins [46].

Lipid rafts have been implicated in a variety of signal transduction mechanisms [37] and other processes, such as cholesterol transport [52] and viral assembly [53]. Because they both sequester and exclude signaling proteins, rafts have been hypothesized to serve as platforms for assembling discrete classes of signaling complexes. Certain signaling proteins, such as heterotrimeric G protein subunits and Src-like kinases localize preferentially to rafts. The reader is directed to a recent review summarizing the published studies relevant to a potential role for cholesterol in PCa, with a focus on cholesterol-rich lipid raft microdomains as potential signaling nodes [54].

Lipid rafts and signal transduction in prostate cancer

Caveolin-1 has been identified as a protein marker associated with PCa progression and hormone-refractory disease [55-57]. Evidence in the literature suggests that caveolin-1 is a

direct mediator of androgen action [33,58], signaling through the PI3K-Akt pathway [59] and metastasis [58] in PCa. Collectively, these studies suggest a role for caveolar lipid rafts in signaling mechanisms relevant to both androgen-mediated and androgen-independent PCa cell growth and survival mechanisms.

While the data on caveolin-1 and PCa point to a role for caveolae in PCa progression, lipid rafts also exist in cells that do not express caveolin proteins [60]. For example, there is an extensive literature on signal transduction by lipid raft-dependent mechanisms in lymphocytes [38], which do not express caveolins. Although the noncaveolar form of lipid raft is less well-defined than are caveolae, the biophysical properties of the two microdomains are similar, if not identical [36]. They also sequester similar classes of proteins [61]. The literature on signal transduction processes mediated by rafts in caveolin-negative cells (e.g., T and B cells [42,62]) suggests that caveolins may not be essential for raft-mediated signaling processes to occur in tumor cells. A further rationale is that, despite the fact that caveolin-1 can be overexpressed in prostate and other malignancies, malignant cells can downregulate caveolin expression and caveolin-1 has been shown to demonstrate "tumor suppressor"-like functions [63,64].

Zhuang et al. were the first to show that cholesterol-rich lipid raft microdomains, in the absence of caveolins, are involved in cell survival signaling in human PCa cells [65]. In that study, the LNCaP PCa cell line was used to demonstrate a requirement for intact plasma membrane lipid rafts in constitutive and epidermal growth factor receptor- (EGFR-) stimulated signaling through the Akt serine-threonine kinase. Raft disruption using cholesterol-binding compounds inhibited both EGF receptor (EGFR) and Akt signaling and induced apoptosis. These effects were reversed by restoring cholesterol to the plasma membrane, indicating that both the signal transduction and cell survival effects of the cholesterol-binding agents were mediated by a cholesterol-dependent process. Cell survival

in LNCaP cells requires continuous signaling through the PI3K-Akt pathway[66], most likely mimicking the situation in many human cancers. The dependence of LNCaP cells on Akt-mediated signaling for survival is emerging as a consistent property of aggressive PCa cells [67,68] and androgen ablation has been shown to upregulate this pathway in model systems [69]. The PI3K/Akt/mTOR pathway is now a major focus of novel drug design for potential clinical application [70,71].

Another recent study by Kim et al. demonstrated a role for caveolin-negative lipid rafts in interleukin-6 (IL-6)-STAT3-mediated neuroendocrine differentiation of LNCaP cells [72]. In that study, phosphorylation of STAT3 by IL-6, and cytokine-stimulated STAT3 translocation to the nucleus, were inhibited by the cholesterol-binding drug, filipin. Isolation of Triton X-100-insoluble raft fractions by sucrose gradient ultracentrifugation demonstrated that the 80 kDa IL-6 receptor localized almost exclusively to the raft membrane fraction. Similarly, STAT3 located in the raft fraction was preferentially phosphorylated by IL-6. IL-6-stimulated increases in expression of neuroendocrine markers were also inhibited by the cholesterol-binding compound. This study carefully examined the cells for the expression of caveolins and demonstrated that they were conclusively negative. Consequently, raft-dependent signaling mechanisms do not require caveolins to elicit physiologically relevant signals in cancer cells. Importantly, IL-6 [73] and STAT3 [74] are PCa marker proteins that can affect signal transduction through the AR [11,75] and neuroendocrine differentiation may be a marker of aggressive disease in PCa as it is in other solid tumor systems [76].

Because the Zhuang et al.[65] and Kim et al.[72] studies suggest that two independent signaling mechanisms of acknowledged relevance to clinical PCa (PI3K-Akt and IL-6-STAT3) are mediated, at least in part, by raft-dependent mechanisms, studies of the raft compartment may provide new insights into the biochemical basis of growth and survival

signals in PCa and other solid tumor systems. If this is true, what role does the AR play in raft-dependent signaling?

Membrane rafts as privileged sites for nongenomic signaling

Treatment of AR-expressing cells with androgen was recently reported by two groups to rapidly activate PI3K-Akt signaling[14,15], most likely by direct binding of AR to PI3K subunits, linking a nongenomic androgen signal to a parallel signal transduction pathway under some control by lipid rafts[65]. AR has been demonstrated to localize to the caveolar form of raft[33] and additional evidence recently obtained by two of us (B.C. and M.R.F.) indicate that AR can reside in non-caveolar (flat) rafts as well (Figure 1). Additional evidence from one of our laboratories (B.C. and M.R.F.) indicates that AR forms complexes with other signaling complexes in this subcellular location in LNCaP cells (not shown). Both caveolar rafts as well as the undifferentiated flat rafts have been identified in a variety of cell types as critical nodes for signal transduction. The observation that AR can transit, and/or stably reside, within membrane rafts suggests the possibility that the protein functions as a signaling intermediate within this subcellular location. A variety of signaling pathways have by now been shown to intersect with the androgenic pathway, including IL-6, IL-4, IGF, and ErbB receptor mechanisms[77]. It remains to be determined whether all of these signaling circuits intersect with the AR exclusively via the classical genotropic pathway. The large number of known AR binding partners[78], most of which at this time do not have a clear functional role in regulating the AR, also suggest possibilities for productive nongenomic signaling processes where AR is a direct participant.

Rafts can be isolated biochemically[33,72,79] and visualized using imaging techniques[80,81]. These and other methods allow a focused approach toward unraveling the mechanism(s) of nongenomic AR action by testing the hypothesis that the lipid raft membrane compartment is a privileged site of nongenomic nuclear receptor signaling. This idea is diagrammed

schematically in Figure 2. As shown in the figure, AR is activated by hormone and a majority of AR protein translocates to the nucleus, resulting in activation of the genotropic signal. However, a minority of the cytosolic AR proteins may also transit to caveolae or, alternatively, to flat, caveolin-negative rafts (our unpublished data) where productive interactions with other signaling proteins may occur. These may affect cellular physiology in ways relevant to tumor cell behavior in vivo, such as growth, resistance to apoptotic signals and motility.

The hypothesis that AR present in rafts can signal independently of its transcription function can now be tested directly by (1) careful, high resolution analysis of the dynamic behavior of AR under conditions of hormonal activation and in response to other AR pathway activators, such as soluble growth factors and cytokines; (2) by the isolation and characterization of signaling complexes containing AR from the non-nuclear subcellular spaces; and (3) by analyzing signals triggered by genomically inactivated AR. Because of the known tendency of the raft compartment(s) to sequester signal transduction complexes, we believe this subcellular location represents a rich potential source of new information on the role of the AR in nongenomic signaling.

Although the significance of nongenomic steroid hormone actions in cancer is still controversial and is being actively debated, the well-known loss of hormonal control of the AR during PCa progression provides a fertile and clinically relevant topic area for investigating these potentially important signaling mechanisms.

References

1 Nelson, W.G. et al. (2003) Prostate cancer. *N Engl J Med* 349 (4), 366-381
2 Jemal, A. et al. (2002) Cancer statistics. *CA Cancer J Clin 2002* 52, 23-47
3 Marker, P.C. et al. (2003) Hormonal, cellular, and molecular control of prostatic development. *Dev Biol* 253 (2), 165-174
4 Heinlein, C.A. and Chang, C. (2002) Androgen receptor (AR) coregulators: an overview. *Endocr Rev* 23 (2), 175-200
5 Balk, S.P. (2002) Androgen receptor as a target in androgen-independent prostate cancer. *Urology* 60 (3 Suppl 1), 132-138; discussion 138-139
6 Chen, C.D. et al. (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 10 (1), 33-39
7 Ueda, T. et al. (2002) Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. *J Biol Chem* 277 (41), 38087-38094
8 Ceraline, J. et al. (2004) Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. *Int J Cancer* 108 (1), 152-157
9 Edwards, J. et al. (2003) Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. *Br J Cancer* 89 (3), 552-556
10 Taplin, M.E. et al. (2003) Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. *J Clin Oncol* 21 (14), 2673-2678
11 Yang, L. et al. (2003) Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells. *Biochem Biophys Res Commun* 305 (3), 462-469
12 Ueda, T. et al. (2002) Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J Biol Chem* 277 (9), 7076-7085
13 Migliaccio, A. et al. (2000) Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *Embo J* 19 (20), 5406-5417
14 Baron, S. et al. (2003) Androgen receptor mediates non genomic activation of phosphatidylinositol 3-OH kinase in androgen sensitive epithelial cells. *J Biol Chem*
15 Sun, M. et al. (2003) Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. *J Biol Chem* 278 (44), 42992-43000
16 Lutz, L.B. et al. (2001) Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. *Proc Natl Acad Sci U S A* 98 (24), 13728-13733
17 Lutz, L.B. et al. (2003) Selective modulation of genomic and nongenomic androgen responses by androgen receptor ligands. *Mol Endocrinol* 17 (6), 1106-1116
18 Hisamoto, K. et al. (2001) Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* 276 (5), 3459-3467
19 Boonyaratanakornkit, V. et al. (2001) Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Mol Cell* 8 (2), 269-280

20 Zhu, Y. et al. (2003) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A* 100 (5), 2231-2236
21 Zhu, Y. et al. (2003) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci U S A* 100 (5), 2237-2242
22 Buitrago, C. et al. (2002) Nongenomic action of 1 alpha,25(OH)(2)-vitamin D3. Activation of muscle cell PLC gamma through the tyrosine kinase c-Src and PtdIns 3-kinase. *Eur J Biochem* 269 (10), 2506-2515
23 Cato, A.C. et al. (2002) Rapid actions of steroid receptors in cellular signaling pathways. *Sci STKE* 2002 (138), RE9
24 Szego, C.M. and Davis, J.S. (1967) Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. *Proc Natl Acad Sci U S A* 58 (4), 1711-1718
25 Schmidt, B.M. et al. (2000) Rapid, nongenomic steroid actions: A new age? *Front Neuroendocrinol* 21 (1), 57-94
26 Georget, V. et al. (1997) Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. *Mol Cell Endocrinol* 129 (1), 17-26
27 Weiner, C.P. et al. (1994) Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci U S A* 91 (11), 5212-5216
28 Kahlert, S. et al. (2000) Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. *J Biol Chem* 275 (24), 18447-18453
29 Stoica, G.E. et al. (2003) Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. *Oncogene* 22 (39), 7998-8011
30 Walker, W.H. (2003) Nongenomic actions of androgen in Sertoli cells. *Curr Top Dev Biol* 56, 25-53
31 Gill, A. et al. (2004) Androgens promote maturation and signaling in mouse oocytes independent of transcription: a release of inhibition model for mammalian oocyte meiosis. *Mol Endocrinol* 18 (1), 97-104
32 Wong, C.W. et al. (2002) Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A* 99 (23), 14783-14788
33 Lu, M.L. et al. (2001) Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. *J Biol Chem* 276 (16), 13442-13451
34 Schlegel, A. et al. (1999) Caveolin-1 potentiates estrogen receptor alpha (ERalpha) signaling. caveolin-1 drives ligand-independent nuclear translocation and activation of ERalpha. *J Biol Chem* 274 (47), 33551-33556
35 Acconcia, F. et al. (2004) S-palmitoylation modulates human estrogen receptor-alpha functions. *Biochem Biophys Res Commun* 316 (3), 878-883
36 Pike, L.J. (2003) Lipid rafts: bringing order to chaos. *J Lipid Res* 44 (4), 655-667
37 Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1 (1), 31-39
38 Dykstra, M. et al. (2003) Location is everything: lipid rafts and immune cell signaling. *Annu Rev Immunol* 21, 457-481
39 Liu, P. et al. (2002) Multiple functions of caveolin-1. *J Biol Chem* 277 (44), 41295-41298
40 van Deurs, B. et al. (2003) Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol* 13 (2), 92-100
41 Harder, T. and Simons, K. (1997) Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr Opin Cell Biol* 9 (4), 534-542
42 Saeki, K. et al. (2003) The B cell-specific major raft protein, Raftlin, is necessary for the integrity of lipid raft and BCR signal transduction. *Embo J* 22 (12), 3015-3026

43 Hur, E.M. et al. (2003) Sensitization of epidermal growth factor (EGF)-induced signaling by bradykinin is mediated by c-Src: Implications for a role of lipid microdomains. *J Biol Chem*

44 Smythe, G.M. et al. (2003) A caveolin-3 mutant that causes limb girdle muscular dystrophy type 1C disrupts Src localization and activity and induces apoptosis in skeletal myotubes. *J Cell Sci* 116 (Pt 23), 4739-4749

45 Mukherjee, A. et al. (2003) Lipid-dependent recruitment of neuronal Src to lipid rafts in the brain. *J Biol Chem* 278 (42), 40806-40814

46 Schlegel, A. and Lisanti, M.P. (2001) The caveolin triad: caveolae biogenesis, cholesterol trafficking, and signal transduction. *Cytokine Growth Factor Rev* 12 (1), 41-51

47 Foster, L.J. et al. (2003) Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A* 100 (10), 5813-5818

48 Munro, S. (2003) Lipid rafts: elusive or illusive? *Cell* 115 (4), 377-388

49 Lai, E.C. (2003) Lipid rafts make for slippery platforms. *J Cell Biol* 162 (3), 365-370

50 Shogomori, H. and Brown, D.A. (2003) Use of detergents to study membrane rafts: the good, the bad, and the ugly. *Biol Chem* 384 (9), 1259-1263

51 Park, D.S. et al. (2002) Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype. *Am J Pathol* 160 (6), 2207-2217

52 Fielding, C.J. and Fielding, P.E. (2001) Cellular cholesterol efflux. *Biochim Biophys Acta* 1533 (3), 175-189

53 Nayak, D.P. and Barman, S. (2002) Role of lipid rafts in virus assembly and budding. *Adv Virus Res* 58, 1-28

54 Freeman, M.R. and Solomon, K.R. (2004) Cholesterol and prostate cancer. *J Cell Biochem* 91 (1), 54-69

55 Yang, G. et al. (2000) Elevated caveolin-1 levels in African-American versus white-American prostate cancer. *Clin Cancer Res* 6 (9), 3430-3433

56 Yang, G. et al. (1998) Elevated expression of caveolin is associated with prostate and breast cancer. *Clin Cancer Res* 4 (8), 1873-1880

57 Mouraviev, V. et al. (2002) The role of caveolin-1 in androgen insensitive prostate cancer. *J Urol* 168 (4 Pt 1), 1589-1596

58 Li, L. et al. (2001) Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells. *Cancer Res* 61 (11), 4386-4392

59 Li, L. et al. (2003) Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. *Mol Cell Biol* 23 (24), 9389-9404

60 Uhles, S. et al. (2003) Isoform-specific insulin receptor signaling involves different plasma membrane domains. *J Cell Biol* 163 (6), 1327-1337

61 Brown, D.A. and London, E. (1997) Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem Biophys Res Commun* 240 (1), 1-7

62 Giurisato, E. et al. (2003) T cell receptor can be recruited to a subset of plasma membrane rafts, independently of cell signaling and attendantly to raft clustering. *J Biol Chem* 278 (9), 6771-6778

63 Lee, H. et al. (2002) Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (-/-) null mice show mammary epithelial cell hyperplasia. *Am J Pathol* 161 (4), 1357-1369

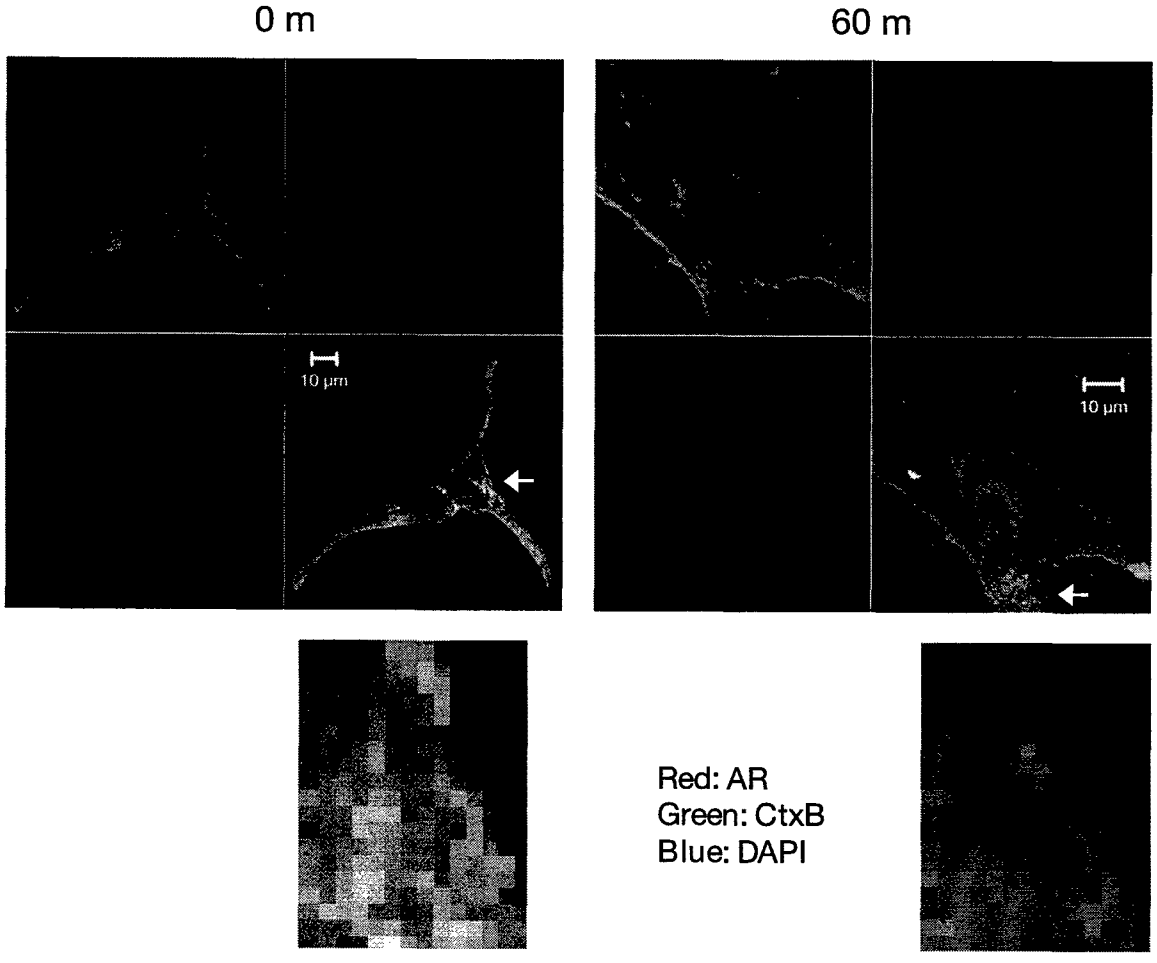
64 Galbiati, F. et al. (2001) Caveolin-1 expression negatively regulates cell cycle progression by inducing G(0)/G(1) arrest via a p53/p21(WAF1/Cip1)-dependent mechanism. *Mol Biol Cell* 12 (8), 2229-2244

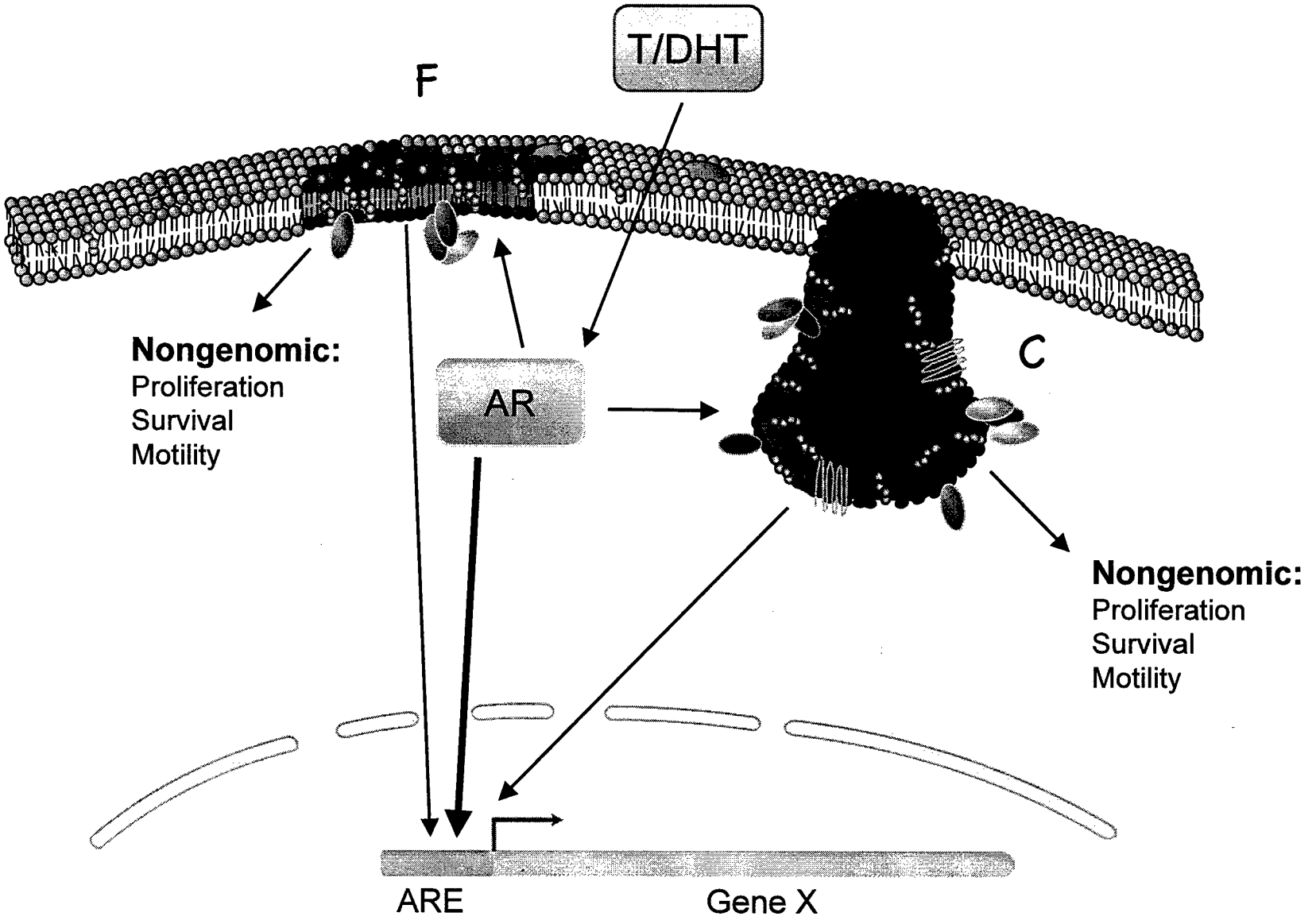
65 Zhuang, L. et al. (2002) Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. *Cancer Res* 62 (8), 2227-2231
66 Lin, J. et al. (1999) The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res* 59 (12), 2891-2897
67 Mousses, S. et al. (2001) Failure of hormone therapy in prostate cancer involves systematic restoration of androgen responsive genes and activation of rapamycin sensitive signaling. *Oncogene* 20 (46), 6718-6723
68 Wang, S. et al. (2003) Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 4 (3), 209-221
69 Murillo, H. et al. (2001) Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* 142 (11), 4795-4805
70 Hill, M.M. and Hemmings, B.A. (2002) Inhibition of protein kinase B/Akt. implications for cancer therapy. *Pharmacol Ther* 93 (2-3), 243-251
71 Graff, J.R. (2002) Emerging targets in the AKT pathway for treatment of androgen-independent prostatic adenocarcinoma. *Expert Opin Ther Targets* 6 (1), 103-113
72 Kim, J. et al. (2004) Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. *Endocrinology* 145 (2), 613-619
73 Nakashima, J. et al. (2000) Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin Cancer Res* 6 (7), 2702-2706
74 Dhir, R. et al. (2002) Stat3 activation in prostatic carcinomas. *Prostate* 51 (4), 241-246
75 Lin, D.L. et al. (2001) Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression. *Clin Cancer Res* 7 (6), 1773-1781
76 di Sant'Agnese, P.A. (2001) Neuroendocrine differentiation in prostatic carcinoma: an update on recent developments. *Ann Oncol* 12 Suppl 2, S135-140
77 Culig, Z. (2004) Androgen receptor cross-talk with cell signalling pathways. *Growth Factors* 22 (3), 179-184
78 Lee, H.J. and Chang, C. (2003) Recent advances in androgen receptor action. *Cell Mol Life Sci* 60 (8), 1613-1622
79 Solomon, K.R. et al. (1998) Determination of the non-ionic detergent insolubility and phosphoprotein associations of glycosylphosphatidylinositol-anchored proteins expressed on T cells. *Biochem J* 334 (Pt 2), 325-333
80 Harder, T. et al. (1998) Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol* 141 (4), 929-942
81 Rotblat, B. et al. (2004) Three separable domains regulate GTP-dependent association of H-ras with the plasma membrane. *Mol Cell Biol* 24 (15), 6799-6810

Figure Legends

Figure 1. Co-localization of AR and lipid rafts in caveolin-negative LNCaP cells. Cells grown in chamber slides were serum-depleted and treated with 1nM R1881 at indicated times. Multi-valent FITC-cholera toxin B conjugate (CtxB) was used to patch and stain the ganglioside raft marker, GM1 (green). AR was detected using Texas Red conjugated anti-mouse (red) secondary antibody. Co-localization of AR and GM1 (yellow) is evident at 0 m but not at 60 m in the blowout panels. Nuclei (blue) are stained with DAPI. Arrows in the lower panels indicate the magnified regions.

Figure 2. Androgenic signaling via cholesterol-rich lipid rafts. The figure depicts the hypothesis that AR-mediated, nongenomic signals arise by a mechanism that involves transit of AR through flat (F) or caveolar (C) membrane rafts. This mechanism is distinct from the genomic signal, which operates separately (bold arrow). Raft-mediated signals may also impinge on androgen-regulated genes in PCa cells, via processes that may or may not involve AR residing in raft domains. The nongenomic biological activities illustrated are hypothetical. ARE=androgen response element. T/DHT=testosterone/dihydrotestosterone.


0 m
60 m
10 µm
10 µm
Red: AR
Green: CtxB
Blue: DAPI



Genomic: Androgen-responsive gene transcription